Diagnostic Cytology

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Dedicated to

Shree Shree Satyananda Giri,
Rini,
Madhumanti,
and
my mother

PREFACE

Cytology is an integral part of pathology curriculum and it is very important to have working knowledge in this area. I designed this book in four sections: general cytology, clinical cytology (exfoliative cytology), laboratory techniques in cytology and fine needle aspiration cytology. I hope that this present book will help immensely all the postgraduate students in pathology, students of cytology (both BSc and MSc), cytology practitioners and all others who are interested in clinical cytology. I have tried to explain various areas of cytology by simple language, tables and illustrations (both line diagrams and microphotographs). All the line diagrams are drawn by me. I hope that these figures are satisfactory. There are multiple boxes in this book which convey the essential and important messages. I will be glad if this book is helpful to the students, teachers and practitioners of cytology.

Pranab Dey

ACKNOWLEDGMENTS

I express my gratitude to Professor Yogesh Chawla, Director, Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, India, for encouraging me to write more books on cytology.

I am proud to work in the Department of Cytology, PGIMER, Chandigarh, India. The excellent set up of this institution helped me a lot.

It is my great pleasure to thank my respected teachers Late Dr Subhash Kumari Gupta and Dr Arvind Rajwanshi, Head of Cytology, PGIMER, Chandigarh. They taught me cytology. I wish to express my sincere thanks to my departmental colleagues Dr Raje Nijhawan, Dr S Radhika and Dr Nalini Gupta with whom I nurture my experience and knowledge of cytology. I am grateful to Professor Kusum Joshi, Head, Department of Histopathology, whose guidance was always my constant support.

During writing this book, I received great support from various persons. Dr Uma Nahar Saikia gave her full cooperation and encouragement in many ways. She particularly helped me to take photography for this book. I am very much thankful to Professor Neelam Verma, Dr Anupam Lal, Dr Ritu Aggarwal, Dr Subhash Chandra Saha, Dr Saroj Sinha, Dr Uttam Mete and Dr Charan Singh Rayat. They donated me generously various photographs for this book.

I am thankful to all my junior residents, senior residents and technical staff in the Department of Cytology. My particular thanks to Dr Adarsh Barwad, who gave his full cooperation in every possible ways. I express my sincere thanks to Mr Rajinder Kumar, our Departmental Photographer, who helped me in taking microphotographs.

Truly speaking, the full credit of the concept of the book goes to my wife Rini Dey. She is the only person who actively encouraged me from the beginning to end of the manuscript preparation. In every step of this book, she helped me immensely.

Lastly, I must admit about the blessing of Great God, the great supreme power, without him a single word cannot be written.

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SECTION 1: GENERAL CYTOLOGY

SECTION 4: FINE NEEDLE ASPIRATION CYTOLOGY

ABBREVIATIONS

Acquired immunodeficiency syndrome	AIDS
Acute lymphoblastic leukemia	ALL
Acute myeloblastic leukemia	AML
Adenoid cystic carcinoma	ACC
Adenomatous polyposis coli	APC
Adenosine triphosphate	ATP
Adrenocortical carcinomas	ACC
Allophycocyanin	APC
Alpha fetoprotein	AFP
American Cancer Society	Acs
Anaphase promoting complex	APC
Anaplastic large cell lymphoma	ALCL
Anaplastic lymphoma kinase	ALK
Androgen receptor	AR
Aneurysmal bone cyst	ABC
Angiomyolipoma	AML
Apoptosis activating factor	APAF
Apoptosis inducing factor	AIF
Argyrophilic nucleolar organizer region	AGNOR
Artificial neural network	ANN
Atypical ductal hyperplasia	ADH
Atypical glandular cells	AGC
Atypical glandular cells of undetermined significance	AGUS
Atypical lipomatous tumor	ALT
Atypical squamous cell	ASC
Atypical squamous cell cannot exclude HSIL	ASC-H
Atypical squamous cell of undetermined significance	ASC-US
Autophagy related gene	ATG
Bacillus Calmette-Guérin	BCG
Bacterial vaginosis	BV
Barrett's esophagus	BE
Basal cell adenoma	BCA
Basal cell carcinoma	BCC
Bayesian belief network	BBN
Bladder tumor antigen	BTA
Bromodeoxyuridine	BrDU
Bronchioloalveolar carcinoma	BAC

Bronchoalveolar lavage	BAL
Burkitt lymphoma	BL
Carcinoembryonic antigen	CEA
CDK inhibitor	CKI
Cerebrospinal fluid	CSF
Cervical intraepithelial neoplasia	CIN
Chromosomal instability	ChIN
Chondrosarcoma	CHS
Chronic lymphocytic leukemia	CLL
Chronic myeloid leukemia	CML
Clear cell sarcoma of kidney	CCSK
Clinical Laboratory Improvement Amendments	CLIA
Cluster of differentiation	CD
Coenzyme A	CoA
Comparative genomic hybridization	CGH
Computerized tomography	CT
Conventional preparation	CP
CpG island methylator phenotype	CIMP
Cyclin-dependent kinase	CDK
Cytokeratin	CKI
Cytomegalovirus	CMV
Cystosarcoma phyllodes	CP
Dense fibrillar component	DFC
Deoxyribonucleic acid	DNA
Desmoplastic small round cell tumor	DSRCT
Diffuse large B cell lymphoma	DLBCL
Disseminated peritoneal adenomucinosis	DPAM
DNA methyltransferases	DNMT
Ductal crcinoma in situ	DCIS
Endodermal sinus tumor	EST
Endoplasmic reticulum	ER
Endoscopic retrograde cholangiopancreatography	ERCP
Endoscopic ultrasound guided FNAC	EUG FNAC
Eosin azure	EA
Epithelial growth factor	EGF
Epithelial membrane antigen	EMA
Epithelial mesenchymal transition	EMT
Epithelioid sarcoma	EST
Epstein-Barr virus	EBV
Estrogen receptor	ER
Ewing's sarcoma	EWS
Fas-associated death domain	FADD
Fibrillar center	FC
Fibrin degradation product	FDP
Fibroadenoma	FAD
Fibroblast growth factor	FGF

Fibrocystic diseases	FD
Fine needle aspiration biopsy	FNAB
Fine needle aspiration cytology	FNAC
Fine needle sampling	FNS
Flow cytometry	FCM
Flow cytometry immunophenotyping	FCI
Fluorescein isothiocyanate	FITC
Fluorescence in situ hybridization	FISH
Focal nodular hyperplasia	FNH
Follicular adenoma	FA
Follicular carcinoma	FC
Follicular center cell lymphoma	FCC
Follicular lymphoma	FL
Follicular neoplasm	FN
Follicular variant of papillary carcinoma of thyroid	FVPTC
Food and drug administration	FDA
Forward light scatter	FCS
Gastrointestinal stromal tumor	GIST
Gastrointestinal tract	GIT
Giant cell tumor	GCT
Giant cell tumor of tendon sheath	GCTTS
Glial fibrillary acidic protein	GFAP
Glucose-transport protein-1	GLUT-1
Golgi complex	GC
G-protein-coupled receptors	GPCR
Granular components	GC
Granulomatous mastitis	GM
Guanosine diphosphate	GDP
Guanosine triphosphate	GTP
Hepatocellular carcinoma	HCC
Herpes simplex virus	HSV
Heterochromatin protein	HP
High grade squamous intraepithelial lesion	HSIL
High mobility group box 1	HMGB1
High power	HP
Histone deacetylase	HDAC
Histone methyltransferase	HMTase
Hodgkin's lymphoma	HL
Homogenously staining region	HSR
Human chorionic gonadotropin	hCG
Human herpes virus	HHV
Human immunodeficiency virus	HIV
Human leukocyte differentiation antigen	HLDA
Human papillomavirus	HPV
Hyaluronic acid	HA
Hybrid capture	HC

Image cytometry	ICM
Immunocytochemistry	IC
Infiltrating duct carcinoma	IDC
Inner nuclear membrane	INM
Insular carcinoma	IC
Internal quality control	IQC
International Society of Urologic Pathologists	ISUP
Intranuclear pseudoinclusions	INI
Intrauterine contraceptive devices	IUCD
Invasive lobular carcinoma	ILC
Lactate dehydrogenase	LDH
Lamin B receptor	LBR
Lamina associated polypeptide	LAP
Langerhans cell histiocytosis	LCH
Large cell carcinoma	LCC
Laser scanning cytometry	LSC
Leiomyosarcomas	LMS
Liposarcoma	LPS
Liquid based cytology	LBC
Loss of heterozygosity	LOH
Low grade squamous intraepithelial	LSIL
Low power	LP
Lupus erythematosus	LE
Lymphoblastic lymphoma	LBL
lymphocytic and/or histiocytic	L & H
Lymphoepithelial sialadenitis	LESA
Lymphoglandular body	LG
Lymphoplasmacytic lymphoma	LPL
Malignant fibrous histiocytoma	MFH
Malignant mesothelioma	MM
Malignant peripheral nerve sheath tumor	MPNST
Malignant round cell tumor	MRCT
Mantle cell lymphoma	MCL
Marginal zone lymphoma	MZL
Matrix attachment region	MAR
May-Grünwald Giemsa	MGG
Mediator of DNA damage checkpoint 1	MDC1
Medium power	MP
Medullary carcinoma	MCL
Messenger RNA	m-RNA
Micro-RNA	miRNA
Micronucleus	MN
Microsatellite instability	MSI
Mitochondria	MT
Mucinous cystic neoplasia	MCN
Mucoepidermoid carcinoma	MEC

Mucosa associated lymphoma	MALT
Multicolor FISH	M-FISH
Multiple endocrine neoplasia	MEN
Myxoid liposarcoma	MLS
Nasopharyngeal carcinoma	NPC
National Health Service, United Kingdom	NHS UK
Natural killer T	NKT
Negative for intraepithelial lesion or malignancy	NILM
Neuroblastoma	NB
Neuron specific enolase	NSE
Nodular lymphocytic predominant HL	NLPHL
Non-Hodgkin lymphoma	NHL
Nontuberculous mycobacteria	NTM
Nuclear matrix proteins	NMP
Nuclear pore complex	NPC
Nucleocytoplasmic	N/C
Nucleolar organizing regions	NOR
Numerical aperture	NA
Oil immersion	OI
Open reading frames	ORF
Oral contraceptive pill	OCP
Orange G	OG
Origin recognition complex	ORC
Outer nuclear membrane	ONM
p53 binding protein 1	53BP1
Pancreatic endocrine tumor	PET
Papillary neoplasia of low malignant potential	PUNLMP
Papillary thyroid carcinoma	PTC
Periodic acid Schiff's	PAS
Peripheral neuroectodermal tumor	PNET
Peripheral T cell lymphoma	PTCL
Peritoneal mucinous carcinomatosis	PMCA
Phosphatidylinositol 3-phosphate	PI3P
Phosphatidylserine	PtdSer
Phycoerythrin	PE
Phyllodes tumor	PT
Placental alkaline phosphatase	PLAP
Plasma cell myeloma	PCM
Platelet derived growth factor	PDGF
Pleomorphic adenoma	PA
Polyclonal carcinoembryonic antigen	p-CEA
Polymerase chain reaction	PCR
Positive predictive value	PPV
Polymorphous low grade adenocarcinoma	PLGA
Pre-replicative	pre-RC
Precursor miRNA	pre-miRNA

Primary effusion lymphoma	PEL
Primary mediastinal large B-cell lymphoma	PMLBCL
Primary miRNA	pri-miRNA
Primitive neuroectodermal tumor	PNET
Progesterone receptor	PR
Programmed cell death	PCD
Proliferating cell nuclear antigen	PCNA
Proliferative breast disease	PBD
Prostate specific antigen	PSA
Psammoma bodies	PB
Reactive lymphoid hyperplasia	RLH
Reed-Sternberg's	RS
Renal cell carcinoma	RCC
Replication licensing factors	RLF
Retinoblastoma	Rb
Reverse transcriptase PCR	RT-PCR
Rhabdomyoma	RM
Rhabdomyosarcoma	RMS
Ribonucleic acid	RNA
Ribosomal DNA	rDNA
Ribosomal RNA	rRNA
Ring finger binding protein	RFBP
Rough endoplasmic reticulum	RER
Scavenger receptors	SR
Sebaceous carcinoma	SC
Single strand conformation polymorphism	SSCP
Sinus histiocytosis with massive lymphadenopathy	SHML
Small cell carcinoma	SCC
Small lymphocytic lymphoma	SLL
Smooth endoplasmic reticulum	SER
Soft tissue sarcoma	STS
Solid and cystic papillary neoplasm	SCPN
Spectral karyotyping	SKY
Spindle assembly check point	SAC
Squamous cell carcinoma	SQC
Squamous intraepithelial lesion	SIL
Standard operating protocol	SOP
Synovial sarcoma	SS
Syringocystadenoma papilliferum	SCAP
Systemic lupus erythematosus	SLE
T cell receptor	TCR
TATA binding protein	TBP
Telomeric repeat amplification protocol	TRAP
Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling	TUNEL
The Bethesda system	TBS
Thyroid stimulating hormone	TSH

Thyroid stimulating immunoglobulin	TSI
Thyroid transcription factor	TTF
Transbronchial needle aspiration cytology	TBNA
Transfer RNA	tRNA
Transitional cell carcinoma	TCC
Trichomonas vaginalis	TV
Tumor necrosis factor-receptors	TNF-receptors
Ultrasonography	USG
Ultrasound-guided FNAC	USG FNAC
Upstream regulatory region	URR
Urothelial carcinoma	UC
Vascular endothelial growth factor	VEGF
Vascular permeability factor	VPF
Visual inspection of cervix with acetic acid	VIA
Wilms' tumor	WT
World Health Organization	WHO

SECTION 1

General Cytology

CHAPTER 1

Cell

Chapter Contents

INTRODUCTION

Cell is the structural and functional unit of life. Human body is made of various types of cells. The fundamental characteristics of these cells are essentially the same. However, during the process of differentiation, the cell acquires many unique morphological and functional properties.

EUKARYOTIC VERSUS PROKARYOTIC CELLS

Cells are mainly classified as prokaryotic or eukaryotic cells. The cells of bacterial and other lower organisms are known as prokaryotic cell. In a prokaryotic cell, deoxyribonucleic acid (DNA) is present within the cytoplasm without any distinct nucleus. The higher animals are made of eukaryotic cells. In eukaryotic cells, DNA is enclosed within the nucleus. In addition, the cell contains mitochondria (MT) and other membrane bound vesicles (**Table 1.1**).

Unlike histology, in cytological examination detailed cell-to-cell relation is often lost. Cytologists study cluster of cells or a single cell for diagnosis. It is essential to know the detailed morphology and function of the cell to understand the alteration of its constituents in reaction to various external and internal stimuli. The various morphological constituents of a cell are highlighted in **Box 1.1** and demonstrated in **Figures 1.1** to **1.3**. The constituents of a cell can be divided into cytoplasmic organelles and nucleus.

CELL MEMBRANE

Cell membrane is a selectively permeable biological membrane that separates the interior of a cell from the external environment.

A *fluid mosaic model* of the cell membrane was first proposed by Singer SJ and Nicolson GL.[1] According to this model, the plasma membrane is just like fluid. The membrane proteins are floating on discontinuous fluid like lipid bilayers. The proteins of the membrane are a set of heterogeneous globular molecules. The highly polar groups are protruding out of the membrane and the nonpolar groups are within the interior portion of the phospholipid membrane. The membrane is described as "mosaic" because it is composed of different types of molecules, such as phospholipids, glycolipids, cholesterol, and proteins (**Fig. 1.4**).[2]

Composition and Structure

The cell membrane is composed predominantly of bilayered phospholipid molecules, proteins, and carbohydrates (**Box 1.2**).

TABLE 1.1: Differentiating features of between eukaryotic and prokaryotic cells

Features	*Eukaryotic cell*	*Prokaryotic cell*
Nucleus	True nucleus with nuclear membrane	No true nucleus
DNA	Circular DNA	Linear DNA
Ribosomes	Complex, five kinds of rRNA	Relatively simple, three kinds of rRNA
Membrane bound organelles	Present	Absent
Cell type	Multicellular	Unicellular
Mitochondria	Present	Absent

BOX 1.1 **Constituents of a cell**

- Cytoplasm
  - Plasma membrane
  - Mitochondria
  - Golgi bodies
  - Rough endoplasmic reticulum
  - Smooth endoplasmic reticulum
  - Centrioles
  - Lysosomes
  - Ribosomes
  - Vacuoles
  - Cytoskeleton
- Nucleus
  - Nuclear membrane
  - Nucleoli
  - Chromatin
  - Nuclear matrix

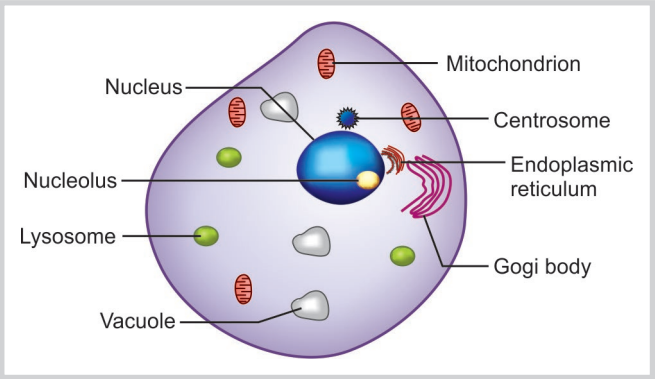


Fig. 1.1: Schematic diagram of eukaryotic cell

- Lipids: There are three classes of lipids: (1) phospholipid, (2) cholesterol and (3) glycolipid. The phospholipids are the predominant type of lipids noted in the cell membrane. There are four varieties of phospholipids: (1) phosphatidylcholine, (2) phosphatidylethanolamine, (3) phosphatidylserine and (4) sphingomyelin. Phospholipids have the hydrophilic or polar ends and hydrophobic or nonpolar ends. In the hydrophilic ends, usually the glycerol molecules combine with serine, choline, or ethanolamine, whereas, in the hydrophobic ends, the glycerol molecule is attached with the long chain fatty acids.

  Hydrophobic ends of the molecules are facing each other and they are away from the cytosol or external environment. Whereas, the hydrophilic ends are facing toward the cytosol (**Fig. 1.5**). At low temperature, the bilayered lipid is just like gel; however, at body temperature, the lipid bilayer is fluid and moving and can exchange their places.

  Good amount of cholesterol molecules are also present in the plasma membrane, and one cholesterol molecule is present for one phospholipid molecule. The cholesterol molecules are embedded within the phospholipid layers.

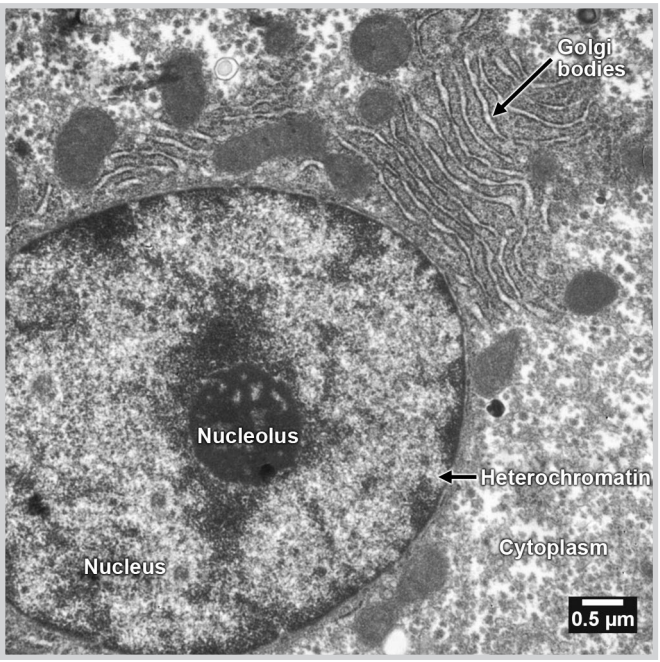


Fig. 1.2: Electron microscopic picture of a cell
(*Courtesy:* Charan Singh Rayat, Department of Histopathology, PGIMER, Chandigarh, India)

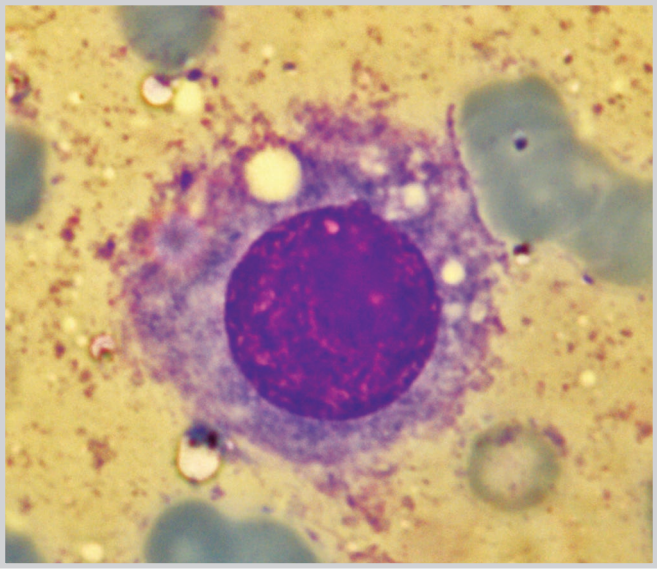


Fig. 1.3: Photomicrograph of a cell with central nucleus having a prominent nucleoli and moderate amount of cytoplasm (May Grunwald Giemsa stain x1000)

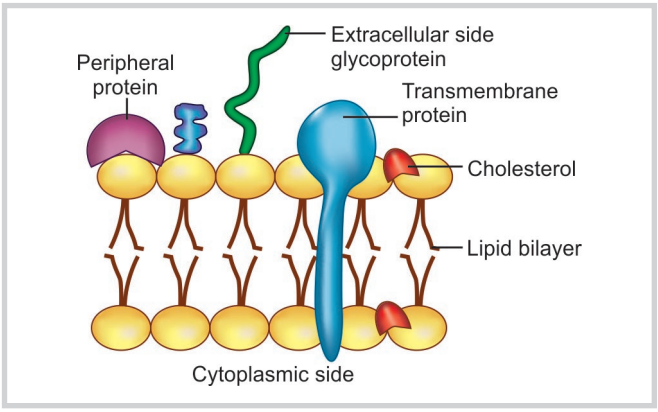


Fig. 1.4: Schematic diagram of lipid bilayered cell membrane

BOX 1.2 Composition of cell membrane

- **Lipids:** Phospholipids, cholesterol, glycolipids
  - Double layer, hydrophilic ends outer side and hydrophobic ends facing each other
- **Carbohydrates:** Glycoproteins and glycolipids
- **Proteins:**
  - Integral protein: Incorporated within the membrane
  - Transmembrane protein: Present through complete breadth
  - Peripheral membrane protein: Present on the inner and outer surface of the membrane

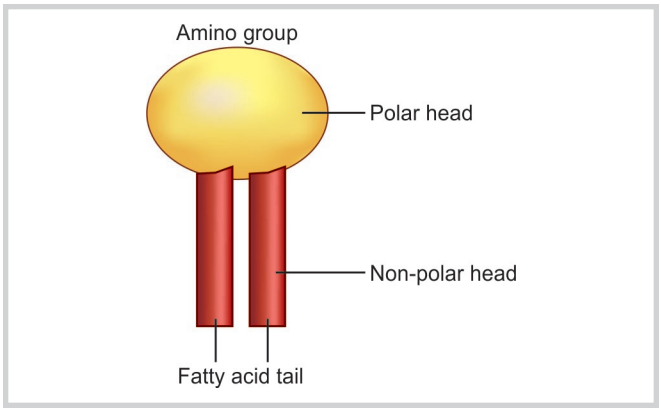


Fig. 1.5: Hydrophilic amino acid tail and hydrophobic fatty acid tail of phospholipid molecule in the lipid bilayered cell membrane

They prevent the mobility of the first few hydrocarbon molecules of the phospholipid and also prevent the crystallization of the hydrocarbon. Thus, cholesterol maintains the fluidity and stability of the membrane.

- Carbohydrate: They are present in the form of glycoproteins and glycolipids. Glycoprotein is the predominant type of carbohydrate and is generally noted on both sides of the membrane. It is involved in cell recognition and protection of the membrane.
- Proteins: Proteins constitute 50% of the membrane. Depending on their positions in the membrane, they may be labeled as: (1) integral protein, (2) transmembrane protein and (3) peripheral membrane protein. Integral proteins are incorporated within the membrane (**Fig. 1.4**). Integral proteins are incorporated within the membrane (**Fig. 1.4**). Transmembrane proteins are the type of integral proteins that traverse through the complete breadth of the membrane. Peripheral membrane proteins are present on the inner or outer surface of the membrane.

Functions

The plasma membrane is biologically active semipermeable membrane with many important functions (**Box 1.3**).

- Cell identity: Plasma membrane encircles the essential component of the cell and acts as a physical barrier between the cell and its surroundings.
- Transport: Plasma membrane is selectively permeable to various substances. As the membrane is hydrophobic in its interior, so it is impermeable to most polar molecules such as Na^+, H^+ and Cl^-. However, the lipid bilayer is permeable to small nonpolar molecules, such as CO_2 and O_2. There are two types of transport that occur through the membrane:
  - Active transport: Energy is needed for this type of transport.
  - Passive transport: No additional energy is needed for this type of transport. This can be channel or transporter protein mediated, facilitated diffusion, or by osmosis. In osmosis, passive transport occurs across the concentration gradient. In case of facilitated diffusion, two types of protein take part in action:

BOX 1.3 Cell membrane—functions

- Cell identity: Maintains cell identity
- Transport: Selectively permeable to various substances
- Signal transduction: Plasma membrane contains many receptors that transport the information via the intracellular signaling proteins
- Cell polarity: Membrane protein complexes are responsible for the polarity of the epithelial cells
- Cell-cell recognition
- Intercellular joining
- Attachment of cytoskeleton and extracellular matrix

  - Transporter proteins: This type of protein alters the conformation of the solutes to be transported and sequentially transports them through the lipid bilayer.
  - Channel protein: This protein forms an aqueous pore across the membrane.
- Signal transduction: Plasma membrane contains many membrane-bound receptors. These receptors bind with signaling molecules and transport the information via the intracellular signaling proteins. The signaling molecules may be soluble, attached to the other cell or may be bound to the extracellular matrix. The three major classes of plasma membrane receptor proteins are involved in signal induction (**Fig. 1.6**):
  - Ion channel-coupled receptors: These receptor proteins are involved in transient opening and closing of the ion channel after binding with the signaling molecules.
  - G protein-coupled receptors (GPCR):[3,4] These GPCRs mediate their actions by transiently binding with a trimeric Guanosine triphosphate (GTP)-binding protein that is also known as G protein (**Fig. 1.6**). The binding of GPCRs and G protein further activates an enzyme or changes the ion permeability of the plasma membrane. The receptor site of GPCR is located toward the extracellular space and the other long chain portion coils the plasma membrane several times.
  - Enzyme-coupled receptors: These receptor proteins are predominantly protein kinases. In their activated form, they phosphorylate the specific types of proteins.
- Cell polarity (**Box 1.4**): Most of the cells in human body are polarized. Cell polarization is studied in the epithelial cell. The epithelial cells have distinct polar distribution, such as luminal surface and basolateral surface facing toward the basement membrane and side of the cell. Various membrane protein complexes are responsible for the polarity of the epithelial cells. Three types of polarity complex proteins are described in the membrane of the epithelial cells: (1) PAR (CDC42–PAR3–PAR6–aPKC), (2) crumbs (Crb–PALS–PATJ), and (3) scribbles (Scrib–Dlg–Lgl). PAR and crumb complexes are involved in the apical polarization and scribble complexes are responsible for basolateral polarization of the epithelial cells. They are also involved in the asymmetric cell division, cell proliferation and cell migration.[5] Asymmetric cell division suppresses cell proliferation.[6] Disruption of the polarity complexes are related with cell proliferation.[7] It is noted that the loss of epithelial cell polarity complexes is related with tumor progression and invasion.[8] So in fact, the membrane polarity complexes behave as tumor suppressor elements.

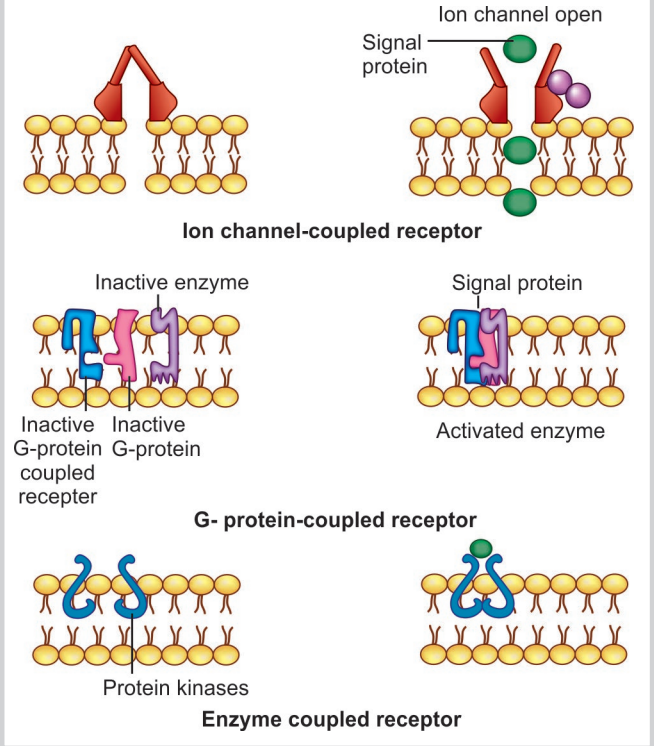


Fig. 1.6: Three types of plasma membrane receptors have been highlighted: ion-channel-coupled receptors, G protein-coupled receptors and enzyme-coupled receptors

BOX 1.4 Cell polarity

- PAR (CDC42–PAR3–PAR6–aPKC), Crumbs (Crb–PALS–PATJ) and Scribbles (Scrib–Dlg–Lgl) are three major polarity complex proteins
- PAR and Crumbs—apical polarity
- Scribbles—basolateral polarity
- Many polarity complex proteins are mutated in cancer
- PAR, Crumbs and Scribbles are indirectly related with asymmetric cell division, cell proliferation and migration
- Cell polarity protein complexes are related with epithelial mesenchymal transition, tumor progression and invasion

Epithelial mesenchymal transition and cell polarity (**Box 1.5**): Epithelial mesenchymal transition (EMT) is an orchestrated series of events in which the epithelial cells loose many of their characteristics and gain the many typical properties of the mesenchymal cells. It is one of the key steps during embryonic development, chronic degenerative fibrosis and cancer metastasis.[9] During the process of EMT, there is loss of polarity of the epithelial cells and the separation of the individual cells that

BOX 1.5 Epithelial mesenchymal transition

The epithelial cells lose their characteristics and gain the properties of the mesenchymal cells
- Key step of embryonic development, chronic degenerative fibrosis and cancer metastasis
- In EMT, there is loss of polarity of the epithelial cells and separation of the individual cells from the basement membrane
- Cancer cells gain motility
- The transformed epithelial cells show increased expression of N-cadherin, vimentin, catenin, and matrix metalloproteinase

help in gaining of cell motility followed by subsequent dispersion of the cells.[10] Evidences suggest that the loss of cell polarity proteins is important in EMT.[11] Decreased expression of cell polarity proteins weakens the apical junctional complexes and therefore induces EMT.[12] E-cadherin is a transmembrane protein that regulates the establishment of the adherens junctions. The intracellular domain of the E-cadherin molecules binds cytosolic catenin and makes a link with actin cytoskeleton. In contrast to epithelial cells, mesenchymal cells do not have any stable intercellular junctions. The mesenchymal cells possess an elongated morphology with front-back symmetry. This facilitates motility and locomotion. Similarly, the loss of E-cadherin in epithelial cells enables them to detach easily and facilitates the dispersion of carcinoma cells from the primary site. In EMT, the transformed epithelial cells show increased expression of N-cadherin, vimentin, catenin, and matrix metalloproteinase.[13,14]

- Cell-cell recognition: Glycolipids and glycoproteins are responsible for mutual cell-cell recognition.
- Intercellular joining: One of the important functions of the plasma membrane is connection between two cells. This is discussed below.
- Attachment to the cytoskeleton and extracellular matrix.

Cilia and Flagella

Cilia and flagella are the mobile extension from the surface of the cytoplasm (**Box 1.6**). Cilia are small, regular and multiple in number, whereas, flagellum is a single slender structure. Cilia are seen on the lining epithelium of the upper respiratory tract and fallopian tube. Each cilium is attached with the thick terminal plate near the apical surface of the cell. The ciliated cells are usually polar and are attached with the basement membrane. The central elongated portion of the cilium is known as axoneme. At the base of the cilium or flagellum is a basal body. The basal body is composed of microtubules. There are total 11 microtubules. In the center, there are two singlet microtubules surrounded by nine triplet microtubules. The shaft of the axoneme consists of nine peripheral doublet microtubules and two central singlet microtubules (**Fig. 1.7**).

Each of the outer peripheral doublet microtubules in the axoneme has a pair of dynein arms that are extended to the adjacent microtubules. These dynein arms help in the movement of the cilium and flagellum. Cilia are usually lost in the cancer

BOX 1.6 Cilia and brush border

Cilia
- Cilia and flagella are the extensions from the surface of cytoplasm
- Cilia are present on the lining epithelium of the upper respiratory tract and fallopian tube
- Basal body is present in the base of cilia
- Axoneme is the central elongated part of cilia
- The shaft of the axoneme: Nine peripheral doublet microtubules and two central singlet microtubules
- Function: Movement of the particles or organism in one direction

Brush border
- Regular finger-like projections on the cell surface of certain specialized epithelial cells
- Commonly seen on the intestinal epithelium and proximal tubular epithelial cells of kidney
- Function: The brush border helps in better absorption of the substances from the large surface area

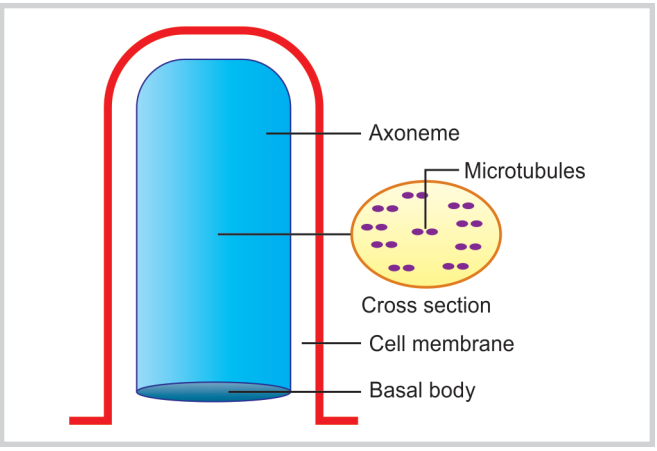


Fig. 1.7: The structure of cilia is shown. The long shaft of axoneme is originated from the basal body. The cross section of axoneme shows nine doublet microtubules and two central singlet tubules

cell originated from the bronchial epithelium. Therefore, the presence of cilia on the cell almost safely excludes the possibility of malignancy.

Function

Cilia help in the movement of the particles or organism in one direction.

Brush Border

The surface of certain specialized epithelial cells covered with multiple microvilli is known as brush border (**Box 1.6**). They are regular finger-like projections on the cell surface about 1 micron

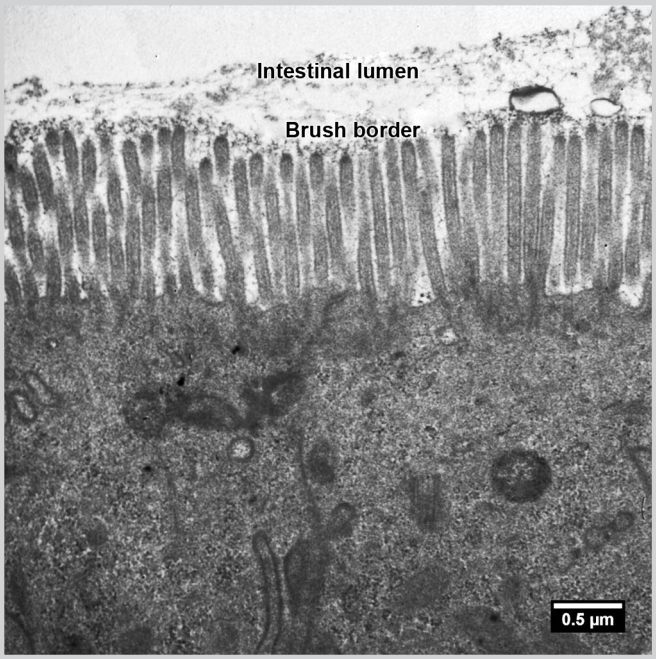


Fig. 1.8: Electron microscopic picture of brush border of intestinal cell (*Courtesy:* Charan Singh Rayat, Department of Histopathology, PGIMER, Chandigarh, India)

in length. Microvilli are commonly seen on the luminal surface of the intestinal epithelium (**Fig. 1.8**) and also on the proximal tubular epithelial cells of kidney. On light microscopy, the microvilli are seen as fuzzy appearance.

Function

The brush border increases the surface area of the cell and helps in better absorption of the substances from the large surface area.

Cell Junction

Cell junction can be classified depending on localization of the junction as follows (**Fig. 1.9**):

- Cell-to-cell
  - Tight junction
  - Adherens junction
  - Desmosomes
  - Gap junction
- Cell-to-matrix
  - Hemidesmosomes.

Tight Junction

This is located in the apical region of the epithelial cells and almost completely seals the gap between the two epithelial cells toward the luminal site. The sealing strands of transmembrane adhesion proteins encircle the apical portion of the plasma membranes of the two cells and hold the membrane tightly. Claudins and occludins are two major transmembrane adhesion proteins.

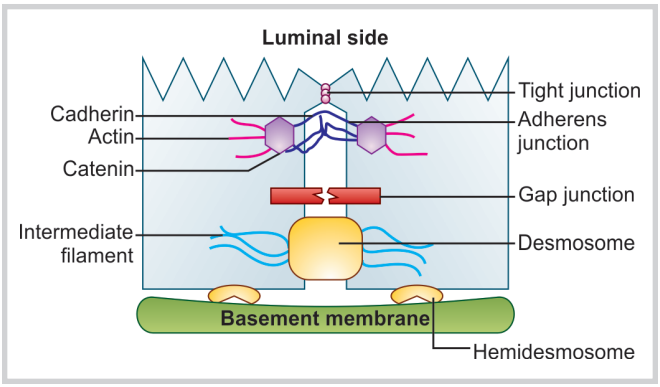


Fig. 1.9: Various types of cell junctions are shown such as tight junction, adherens junction, gap junction, desmosome, and hemidesmosome

Function

There are two major functions of the tight junctions:

1. Tight junction closes the gap between luminal side and the intercellular space. This helps in effective transport of substances from luminal side of the cell to the extracellular fluid compartment.
2. Tight junction acts as a barrier and prevents the drift of the apical membrane proteins to the basal region and *vice versa*.

Adherens Junction

The adherens junction holds the two cells together and confers mechanical strength. Adherens junction is made of cadherin, catenin, and intracytoplasmic actin filaments. Altogether they form adhesion belt-like structure.

Function

To provide cell-to-cell adhesion and mechanical strength.

Desmosomes

These are button-like spots which connect the plasma membrane of two cells together. Desmosomes are linked to the intermediate filaments. The type of intermediate filament depends on the type of cell.

Function

The desmosomal junction provides tensile strength and rigidity of the tissue.

Gap Junctions

These are intercellular channels that connect two adjacent cells. In gap junctions, the two plasma membranes are connected by the transmembrane proteins known as connexins.

Function

There are continuous channels between the two adjacent cells and therefore the cells can rapidly share small molecules and ions.

With the help of the gap junctions the action potential can rapidly travel among a group of cells without any neurotransmitter.

Hemidesmosomes

Hemidesmosomes connect the cell with the basal lamina. Hemidesmosomes are composed of keratin filaments, dystonin, plectin, integrin, collagen XVII, and laminin. In hemidesmosome, integrin binds with keratin in cytoplasmic side by dystonin and plectin. It also binds with collagen XVII and laminin toward basal lamina side.

Function
It attaches cell with the basal lamina.

CYTOPLASMIC ORGANELLES

Endoplasmic Reticulum

Endoplasmic reticulum (ER) is a tubular and cistern-like space and vesicular structure folded within the cytoplasm. An ER generally connected with cell membrane to nuclear membrane (**Box 1.7**). The cisterns are membrane-like long flat spaces which are straight, whereas tubules are irregularly branched structures. The ER contains fluid with many enzymes and proteins. There are two types of endoplasmic reticulum: (1) rough endoplasmic reticulum (RER) and (2) smooth endoplasmic reticulum (SER). The membrane of the RER is continuous with the membrane of SER (**Figs 1.10** and **1.11**).[15,16]

Rough Endoplasmic Reticulum

They are tightly packed parallel bundles of cistern-like spaces which are beaded in appearance due to ribosome particles attached to the surface of RER. The ribosomes are bound with RER by a receptor known as ribophorin.

Function
It is the site of synthesis of secretory protein synthesis and lysosomal enzymes.

BOX 1.7 Endoplasmic reticulum

- Tubular and cistern-like spaces and vesicular structures folded within the cytoplasm.

Rough endoplasmic reticulum:
- Membrane of RER is continuous with outer layer of nuclear membrane
- Beaded in appearance due to ribosome particles attached to the surface
- Function: Synthesis of secretary protein and lysosomal enzymes

Smooth endoplasmic reticulum:
- Connected with Golgi apparatus and plasma membrane
- Function: Synthesis of lipids

Smooth Endoplasmic Reticulum

The SER predominantly contains tubules and vesicles. SER is connected with Golgi apparatus and plasma membrane.

Function
Synthesis of lipids.

Golgi Complex

Golgi complex (GC) are stacks of membrane-bound cistern-like spaces within the cytoplasm arranged in a polarized fashion. Each stack of GC has four parts (**Box 1.8**, **Figs 1.2** and **1.12**):

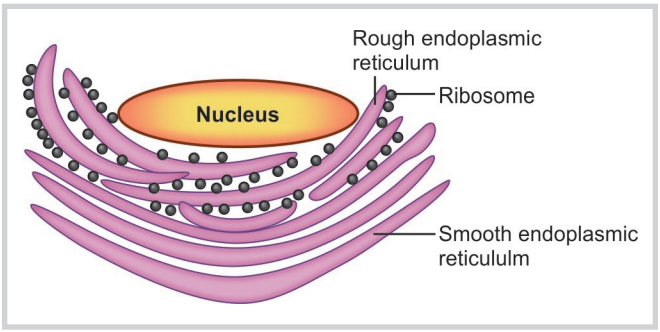


Fig. 1.10: Rough endoplasmic reticulum is studded with ribosomes. The membrane of rough endoplasmic reticulum is continuous with outer layer of nuclear membrane

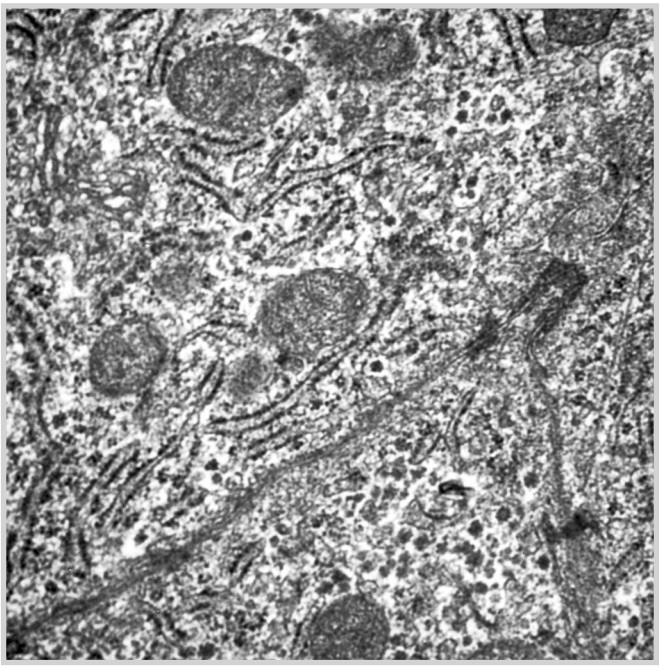


Fig. 1.11: Electron microscopic picture of rough endoplasmic reticulum (*Courtesy:* Uma Nahar Saikia, Additional Professor, Department of Histopathology, PGIMER, Chandigarh, India)

BOX 1.8 Golgi complex

- Membrane-bound cistern-like spaces arranged in a polarized fashion
- Types: Cis Golgi network, endo-golgi and medial Golgi, and trans-Golgi network
- Functions:
  - Processing of the protein received from RER. N-linked and O-linked glycosylation of protein and lipids
  - Calcium storage
  - Platform of various cell signaling

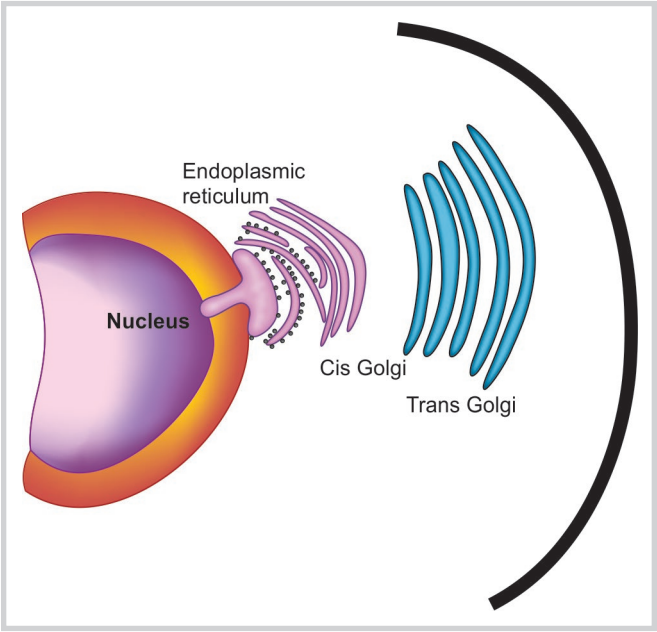


Fig. 1.12: Golgi complex has cis, trans, endo and medial parts. Cis Golgi faces towards the nucleus and trans Golgi towards the cell membrane

- **Cis Golgi network:** Cis Golgi network is the concave surface of the stack of GC that faces towards the RER and small transfer vesicles. Cis Golgi network receives the initial protein from ER.
- **Endo Golgi and medial Golgi:** These are the middle parts of GC and most of the proteins are modified here.
- **Trans-Golgi network:** This is the convex surface of the stack of the GC. Trans-Golgi network is associated with large secretory vesicles and final transport of the protein.

Function

- The main function of the GC is chemical processing of the protein received from RER followed by packaging and transfer.
- Along with classical "protein trafficking", there are many other novel functions of GC such as entry of the cell to mitotic check point, calcium homeostasis and cytoskeletal organization.[17]
- Protein modification: N-linked and O-linked glycosylation of protein and lipids occur in the GC.[18]

BOX 1.9 Mitochondria

- Power house of the cell
- Mitochondria are double membrane-bound structures
- Parts: Outer membrane, inner membrane, intermembranous space, cristae, mitochondrial matrix
- Outer membrane
  - Rich in porin
  - Small ions and proteins can pass
  - Connected with endoplasmic reticulum
- Inner membrane
  - Rich in cardiolipin
  - Impermeable to proton, ion and electrons
  - Rich in: (1) ATP synthase (2) Respiratory chain protein complexes, e.g. NADH dehydrogenase complexes, Cytochrome b-c1 and cytochrome oxidase and (3) transport protein complexes
- Matrix: Rich in enzymes of citric acid cycle and mitochondrial DNA
- Functions: Main function of mitochondria is energy production in the form of ATP synthesis.
  - Citric acid cycle: Main reactions of citric acid cycle occur in mitochondria
  - Electron transport: During oxidative phosphorylation, a series of electron transport reactions occur
  - Calcium storage: Plays an important role in calcium homeostasis
  - Cell cycle: Signaling platform for cell cycle progression
  - Apoptotic death: Plays a key role in apoptosis by activating proapoptotic enzymes

- Protein transport: GC receives the neosynthesized protein from the ER and transport to their respective destination. The cargo proteins are first modified and then they are transported by GC. The mechanism of transport of cargo proteins is not exactly known. However, there are two theories:
  - *Vesicular transport model theory:* The cargo protein is transported by an anterograde way with the help of vesicles that bud from one cisterna and then fuse to the next one.
  - *Cisterna maturation model:* In this model, it is assumed that the Golgi cisterns are formed *de novo*, progressively mature and finally dissipate.[19]
- Calcium storage: GC is the most important site of intracellular calcium storage and can also release Ca^{2+} in case of agonistic stimulation.[20]
- Platform of different cells signaling: GC acts as a platform of different signaling events within the cell. In addition to receiving initial signal, GC can also induce a cascade of signal transduction.

Mitochondria

These are 0.5–1 micron diameter organelle considered as the power house of the cell, as they are the major source of energy (**Box 1.9**). Mitochondria are richly localized in that part of the cell

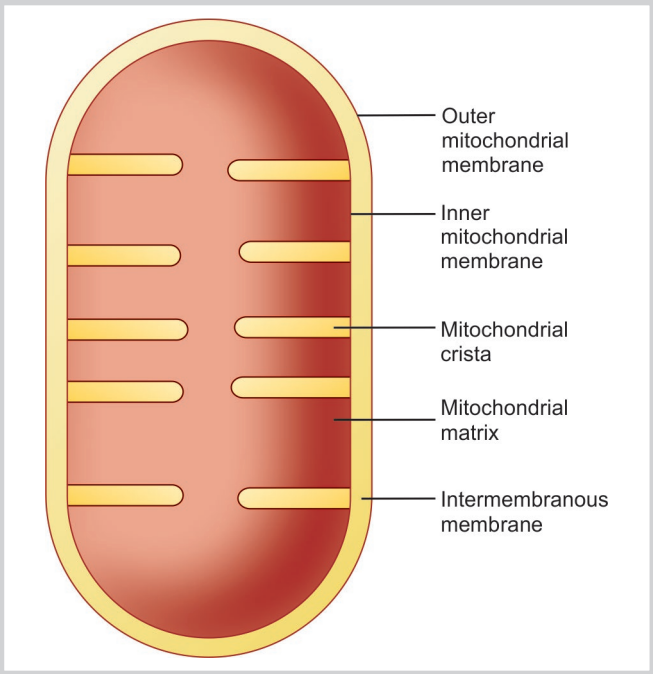


Fig. 1.13: Double membrane-bound structure of mitochondrion with outer and inner membranes

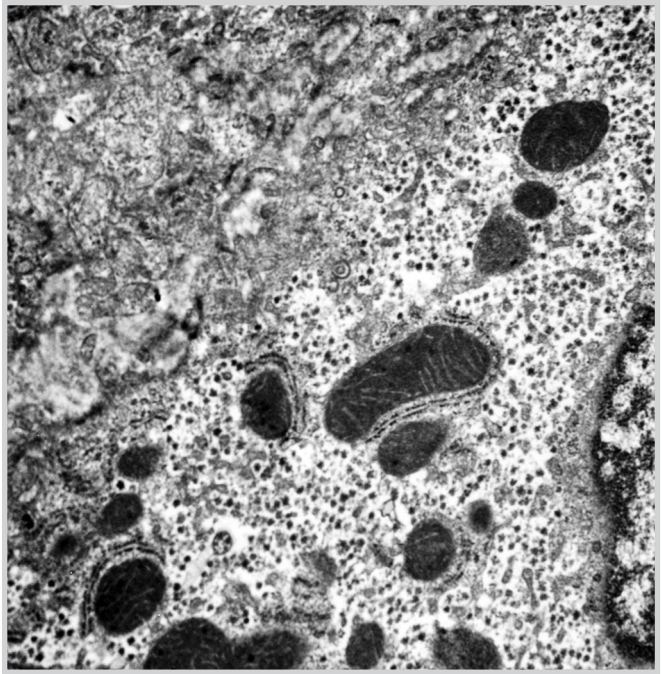


Fig. 1.14: Electron microscopic picture of mitochondria (*Courtesy:* Uma Nahar, Saikia, Additional Professor, Department of Histopathology, PGIMER, Chandigarh, India)

which requires energy, such as interfibrillar space in the striated muscle and in the middle part of the sperm. Other than nuclear DNA, MT has its own independent DNA and this is the unique feature of MT.

Structure

Mitochondria are double membrane-bound structures, which consist of following parts (**Figs 1.13** and 1.**14**):

1. Outer membrane
2. Inner membrane
3. Intermembranous space
4. Cristae
5. Mitochondrial matrix.

- Outer membrane: This is composed of phospholipid bilayers. The outer membrane of MT is rich in porin, a variety of integral protein. This forms a through and through aqueous channel in the outer membrane. Therefore, the small ions and proteins can pass through the outer membrane easily. The outer membrane is also connected with ER by mitochondria-associated endoplasmic reticulum.
- Inner membrane: This is the inner phosphopolipid bilayer of the MT. The inner membrane is rich in double phospholipid known as cardiolipin. The cardiolipin possesses four fatty acids rather than two, and the presence of cardiolipin makes the inner membrane impermeable to proton, ion, and electrons. In certain regions, the outer and inner membrane joins together known as contact sites and makes a passage of the proteins and small molecules from the cytoplasm to the matrix space. The inner membrane contains the large number of lollipop-like structure with small stalk attached to the inner membrane and globular region in the matrix. These globular regions contain protein complex of ATP (Adenosine triphosphate) synthase. The inner membrane contains three types of enzymes: (1) ATP synthase (2) the respiratory chain protein complexes such as NADH dehydrogenase complexes, Cytochrome b-c_1 and Cytochrome oxidase, and (3) transport protein complexes.
- Intermembranous space: This is the minute space between the inner and outer membrane of the MT. The concentration of small molecules is same in both cytoplasm and intermembranous space.
- Cristae: These are the small shelf-like folds of the inner membrane. This makes the larger space in the inner membrane to retain more enzymes.
- Matrix: It is the innermost space of MT encircled by the inner membrane. The matrix consists of dense fluid that is rich in viscosity. Matrix is rich in enzymes of citric acid cycle and also contains *mitochondrial DNA*. Mitochondrial DNA is unique to MT, and it is considered as separately developed during evolution. In sexual reproduction, MT is exclusively inherited from mother and so MT DNA is of maternal origin. MT DNA is circular DNA. This is responsible for the formation of selected MT protein, ribosomal ribonucleic acid (rRNA), and transfer RNA (tRNA). Replication of mitochondrial DNA is not limited to the S phase of the cell cycle, and this may occur in any phase of the cell cycle.

Function

Main function of MT is energy production in the form of ATP synthesis. However, it is also involved in other important functions, such as calcium storage, cell death, etc.[21]

- Citric acid cycle: The essential enzymes in citric acid cycles are located in the mitochondrial matrix, and the main reactions of citric acid cycle happen in the mitochondrial matrix. Initial oxidative breakdown of glucose occurs in the cytoplasm by the process known as glycolysis. The glucose is converted to pyruvate, which is transported to the mitochondrial matrix. This is further oxidized to acetyl coenzyme A (CoA). Fatty acid also enters into the mitochondrial matrix and is oxidized to acetyl CoA. Glucose to acetyl CoA is the basic ingredient of citric acid cycle. The main end-products of citric acid cycle are CO_2, $FADH_2$, and NADH. $FADH_2$ and NADH further help in the production of ATP.
- Electron transport: During the oxidative phosphorylation, a series of electron transport reactions occur in the inner mitochondrial membrane. Electrons from NADH enter to flavin mononucleotide and then through a series of complex proteins to molecular oxygen. The energy released in these reactions is used to generate ATP from ADP.
- Calcium storage: Mitochondria can store calcium and play an important role in calcium homeostasis.[22]
- Cell cycle: Mitochondria play as a signaling platform for cell cycle progression. AMP-activated protein kinase is activated in the MT which helps in phosphorylation of serine 15 of p53 protein. This prevents the degradation of this protein p53 and, which subsequently helps in cell cycle arrest in DNA damage.[23]
- Apoptotic death: Mitochondria play a key role in apoptosis. During the process of apoptosis MT releases cytochrome c, which activates Apaf-1 and ultimately caspase 9 is activated. Activated Caspase 9 breaks DNA in small pieces causing apoptosis. Release of Cytochrome c from the MT membrane is induced by pro-apoptotic BCl-2 family members.[24]

Ribosome

Ribosome is the site of protein synthesis (**Box 1.10**).

Structure

Ribosomes are small 25–30 nm particles present in the cytoplasm. They are present both as free ribosomes in cytosol and also in the membrane-bound form attached with ER and thus forming RER. The ribosome is made up of rRNA and proteins. The ribosome is classified according to the sedimentation coefficient in ultracentrifugation. The eukaryotic ribosome has two units, a smaller 40S and a larger 60S subunit. In its inactivated form, the two subunits are detached; however, when the ribosome is engaged in protein synthesis both the units are attached together. Small subunit has the binding site for mRNA and tRNA. Some rRNA of the larger subunit has enzymatic activity to catalyze the peptide bond. These rRNA are known as the ribozyme (**Fig. 1.15**).

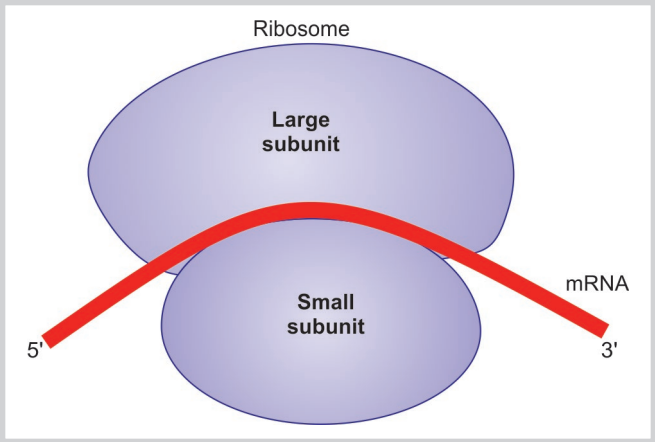


Fig. 1.15: Schematic diagrams of two subunits of ribosome

BOX 1.10 Ribosomes

- Sites of protein synthesis
- Present as free ribosomes in cytosol and membrane bound form attached with endoplasmic reticulum
- Made up of rRNA and proteins
- Classified according to the sedimentation coefficient in ultracentrifugation
- A ribosome has two units: a smaller 40S and larger 60S
- Small subunit has the binding site for mRNA and tRNA
- rRNA of the larger subunit has enzymatic activity to catalyze the peptide bond.
- Function: Decode the information from mRNA and help the molecules of tRNA to assemble the particular amino acids to make a protein.

Function

Ribosomes play a vital role in protein synthesis by decoding information from mRNA and then help molecules of tRNA to assemble particular amino acids to make a protein.

Lysosomes

These are 0.2–0.4 micron small membrane-bound vesicles present in the cytoplasm (**Box 1.11**). They contain near about 40 acid hydrolytic enzymes. Lysosomal enzymes are synthesized in the ER and transported in GC. The lysosomal enzymes and membrane of the lysosome are finally synthesized in the trans-Golgi network and are carried to the endosome by clathrin-coated transport vesicles. The final lysosomal vesicles are synthesized in the late endosomal intermediate (also known as endolysosome).

There are two types of lysosomes: (1) primary lysosome (no morphological sign of hydrolytic enzymes) and (2) secondary lysosomes (when lysosome fuses with other phagocytic vesicles of an organism, it shows enzymatic activities, and this lysosome is known as secondary lysosomes).

Function

Lysosome contains acid hydrolytic enzymes such as lipase, amylase, protease and nuclease. These enzymes are activated in the acid environment. The lysosomal enzymes digest the

BOX 1.11 Lysosome

- Lysosomal enzymes are synthesized in the endoplasmic reticulum and transported in Golgi complex
- Primary lysosome: No morphological sign of hydrolytic enzymes
- Secondary lysosomes: Lysosome fuses with other phagocytic vesicles or organism and shows enzymatic activities
- Functions:
  - Lysosome contains acid hydrolytic enzymes, such as lipase, amylase, protease and nuclease. Lysosomal enzymes degrade the protein and carbohydrate components of the organism.

macromolecules, destroy the microbes and remove the other cytoplasmic organelle such as mitochondria. The foreign organisms enter the cytoplasm as phagocytic vesicles. Lysosome fuses with the phagosome and releases acid hydrolytic enzymes, which degrade the protein and carbohydrate components of the organism. The lipid component is more resistant to digestion and may remain as the residual body. At times, lysosome fuses with the nonfunctioning MT or fragments of RER to clear these substances from the *autophagic* vacuoles in the cytoplasm. When these autophagic vacuoles remain persistently in the cytoplasm, they accumulate pigment known as lipofuscin.

Peroxisome

These are tiny vesicles of 0.2–1 micron in size. They are synthesized from RER. Peroxisomes contain many oxidative enzymes. The enzymes in the peroxisome break down fatty acid by beta oxidation and generate acetyl CoA and H_2O_2. Acetyl CoA is involved in various energy producing metabolic processes. The hydrogen peroxide helps kill the various organisms. Excess hydrogen peroxide is further degraded by catalase enzyme of peroxisome into water and oxygen.

Cytoskeleton

Cytoskeleton is the meshwork of protein filaments within the cytoplasm of the cell that maintains the shape of the cell along with other important functions, such as cell movement, cell contraction and maintaining cell polarity (**Box 1.12**). There are three components of the cytoskeleton: (1) microfilament, (2) microtubules and (3) intermediate filaments.

Microfilament

It is also known as *actin filament*. These are the thinnest filaments and present either as a bundle or as fine network within the cytoplasm. The actin filament is composed of globular G-actin which is polymerized and forms a long chain of F-actin (**Fig. 1.16**). Most of the G-actin is bound with small proteins such as profilin and thymosin. Binding of these small proteins prevents the polymerization of G-actin. Actin filament binds with filamin and makes a robust supporting meshwork just underneath the plasma membrane known as cell cortex. There are three types of actin: (1) alpha actin, (2) beta actin and (3) gamma actin. Alpha actin is present in muscle, and other two forms of actin are present in nonmuscular cells.

BOX 1.12 Cytoskeleton

Three types: microfilament, microtubules, and intermediate filaments.

- **Microfilament (actin filament)**
  - Fine network of thin actin filament within the cytoplasm
  - Globular G-actin which is polymerized and forms a long chain of F-actin
  - Forms a robust supporting meshwork just underneath the plasma membrane known as cell cortex
  - Functions: Maintains shape of the cell, cell contraction, cell movement, phagocytosis, transport of vesicles in the cytoplasm

- **Microtubules**
  - The basic constituents: alpha and beta tubulin
  - Alpha and beta tubulin is arranged alternatively to form a protofilament
  - The protofilament of tubulin is polar as one end is formed by beta tubulin and other end is formed by alpha tubulin
  - Functions: Intracellular transport, mitotic spindle movement, movements of cilia and flagella

- **Intermediate filament**
  - The diameter of intermediate filaments is in between the microfilament (7 nm) and microtubules (25 nm)
  - The polypeptide is alpha helix with 310–350 amino acids
  - Two such alpha helix monomer coils to form a dimer and the two dimers coil in a staggered antiparallel fashion to form a tetramer. Eight such tetramers twist in a rope like manner to form an intermediate filament
  - Types of intermediate filaments: acidic and neutral basic keratin, vimentin, desmin, glial fibrillary acidic protein, peripherin, neurofilaments, lamin and nestin
  - Functions: Support of the cytoskeletal structure, chromatin organization of the nucleus

Functions

- Actin maintains shape of the cell
- Actin binds to the myosin and helps in contraction of muscle fibers
- Actin can shorten its length and helps in the movement of the cell
- Phagocytosis or pinocytosis is helped by actin
- Actin helps in the transport of various vesicles within the cytoplasm.

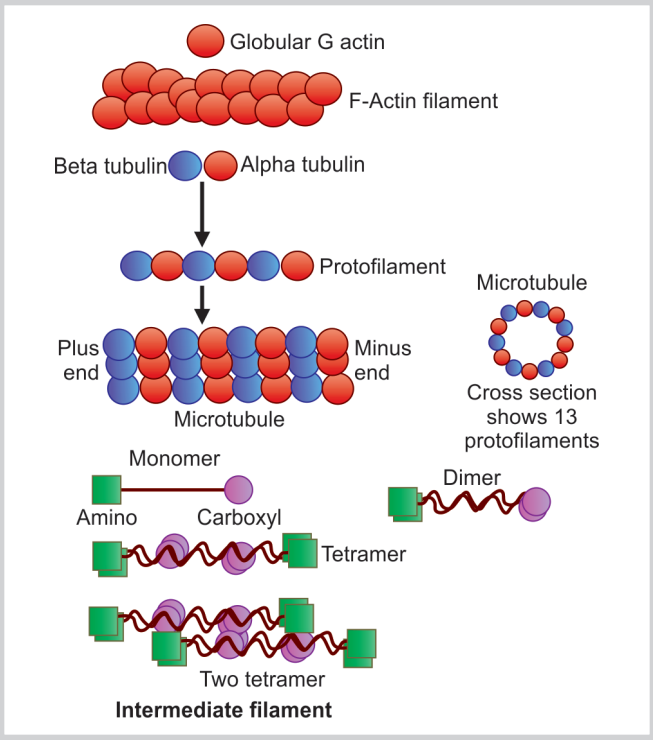


Fig. 1.16: Three types of cytoskeletal structures: actin filament, microtubules and intermediate filament

Microtubules

Microtubules are long, straight, hollow rigid tubules of 25 nm diameter. These are dynamic fibers, as they are always in the process of assembling and disassembling. The microtubules constitute mitotic spindles, centrioles, cilia and flagella. The basic constituents of the microtubules are alpha (α) and beta (β) tubulin. These tubulins are arranged alternatively to form a protofilament (**Fig. 1.16**). GTP is tightly bound with α tubulin and resistant to hydrolysis whereas it is loosely bound with β tubulin and can be separated by hydrolysis. The protofilament of tubulin is polar as one end is formed by β tubulin and other end is formed by α tubulin. The β end of the tubulin protofilament is plus end as the growth and shrinkage of this end is rapid. The opposite α tubulin end is known as the minus end. In each microfilament, there are a total of 13 protofilaments. These protofilaments are parallel in position. All the plus ends or growing ends of the protofilaments are in one direction.

Functions

The main functions of the microtubule are:

- Intracellular transport: Microtubules help in the transport of vesicles containing proteins from the GC to plasma membrane.
- Mitotic spindle movement: The mitotic spindles are formed by microtubules. The chromatids are separated and pulled to each daughter cell nucleus by the mitotic spindles formed by microtubules.
- Movements: Movements of cilia and flagella are done by the microtubules.

Centrosome

It is small round body located near the nucleus in the interphase cell. This is also known as microtubule organizing center. The microtubules are attached with the centrosome by their minus ends and they radiate from the centrosome in a star-shaped manner. Centrosome consists of a pair of centrioles arranged in L-shaped manner surrounded by the amorphous matrix material known as centrosome matrix or pericentriolar material. Centrosome matrix material takes the main role in the development of the microtubule. Centrioles are the basal bodies of cilia or flagella. During mitosis, the centrosome duplicates and each one contains one pair of centrioles. From each of the centrosomes, microtubules radiate and form a complete mitotic spindle.

Intermediate Filament

Intermediate filaments have an average diameter of 10 nm. The name of the intermediate filaments is such because the diameter of intermediate filaments is in between the microfilaments (7 nm) and microtubules (25 nm). The individual polypeptide of intermediate filaments is an alpha helix with 310–350 amino acids. It has N and C terminals. Two such alpha helix monomers coil with each other to form a dimer. Both the N and C terminals are in same direction in this monomer. Two dimmers then coil in a staggered antiparallel fashion to form a tetramer. Eight such tetramers twist in a rope-like manner to form an intermediate filament. Therefore, in a cross section of intermediate filament, there are 32 alpha helix coils.

Types[25]

There are a total six types of intermediate filament (**Table 1.2**):

- Type I and type II: Acidic and neutral basic keratin. Type I keratin is acidic and type II keratin is basic in nature. They include a good number of epithelial and hair keratins.
- Type III: There are four varieties of type III intermediate filaments. They are:
  - Vimentin: This is widely expressed in mesenchymal cells and a variety of other cells such as leukocytes, vascular endothelial cells and some epithelial cells.
  - Desmin: Desmin is noted in the skeletal and cardiac muscle fibers.
  - Glial fibrillary acidic protein (GFAP): This is expressed in astrocytes and other glial cells.
  - Peripherin: It is noted in the peripheral neurons, such as neurons of the dorsal root ganglia, sympathetic ganglia and cranial nerves.
- Type IV
  - Neurofilaments: Neurofilaments are classified according to their sizes as NF-L (light, 62 kDa), NF-M (medium 102 KDa) and NF-H (heavy 112 kDa). They are expressed in the mature neurons.
  - Alpha internexin: These are found in developing central nervous system.
- Type V
  - Lamin: They are found in the nucleus of the cell as lamin A, lamin B and lamin C. Lamin is noted as a proteinaceous

TABLE 1.2: Intermediate filaments

Type	*Varieties*	*Location*	*Molecular weight (Da)*	*Function*
I	Acidic keratin (11 epithelial keratin, four hair keratin)	Epithelial cells	40,000–70,000	Tensile strength
II	Basic keratin (8 epithelial keratin and four hair keratin)	Cells of Hair and nail	40,000–70,000	Tensile strength
III	Vimentin	Mesenchymal cells, leukocytes, vascular endothelial cells and some epithelial cells	54,000	Support the cytoplasmic membrane and helps in holding the various organelles in proper position
	Desmin	Skeletal and cardiac muscle fibers	53,000	Helps in stabilizing sarcomeres of the contracting muscle cells
	Glial fibrillary acidic protein	Astrocytes and other glial cells	50,000	Supports the glial cells
	Peripherin	Neurons of the dorsal root ganglia, sympathetic ganglia and cranial nerves	56,000	Supports the neurons
IV	Neurofilaments (NF)	Mature neurons		They form the cytoskeleton of dendrites and axons.
	NF—Light		62,000	
	NF—Medium		102,000	
	NF—High		110,000	
V	Lamin A, lamin B, and lamin C	Nuclear envelope	65,000–75,000	Control of assembly of the nuclear envelope during mitotic event and chromatin organization
VI	Nestin	Stem cells of the central nervous system and in developing skeletal muscle	2,00,000	

structural meshwork underneath the nuclear membrane and also found within the nucleoplasm. The meshwork of lamin underneath the nuclear membrane acts in chromatin organization and gene expression.

- Type VI
  - Nestin: Nestin is expressed in proliferating stem cells of the central nervous system and in developing skeletal muscle.[26]
- Unclassified
  - Filensin: It is expressed during the differentiation of the vertebrate lens epithelial cells.

Functions

- Supporting the cytoskeleton structure: Intermediate filaments are more stable than microtubules and microfilaments and provide good support and tensile strength to the cytoskeleton of the cell. Desmin links myofibrils of the striated muscles. GFAP supports the glial structure, and neurofilaments support the cytoskeleton of the axons and dendrites.
- Chromatin organization: Nuclear lamin plays an important role in chromatin organization of the nucleus. They also play a role in control of assembly of the nuclear envelope during the mitotic event.

NUCLEUS

Nucleus is the central processing unit of the cell and acts as the controlling center of the cell (**Box 1.13**). The important components of the nucleus are:

- Nuclear envelope and pore

BOX 1.13 **Nuclear membrane**

- Nuclear membrane:
  - Outer nuclear membrane (ONM)
  - Inner nuclear membrane (INM)
  - Perinuclear space
- The nuclear lamina is located in the nuclear side of the INM
- ONM is continuous with the endoplasmic reticulum
- INM contains various integral proteins such as lamin B receptor, lamina associated polypeptide, emerin, and ring finger binding protein
- Functions
  - Physical barrier between cytoplasm and nucleus
  - Helps in chromatin remodeling and gene expression

- Nuclear matrix
- Nuclear chromatin
- Nucleoli.

Nuclear Envelope

Nuclear envelope is the barrier which separates the nucleus from the cytoplasm. It is composed of three parts:

1. Nuclear membrane
2. Nuclear pore
3. Nuclear lamina.

Nuclear Membrane

The nuclear membrane is further divided into outer nuclear membrane (ONM), inner nuclear membrane (INM), and perinuclear space (**Figs 1.17** and 1.**18**).

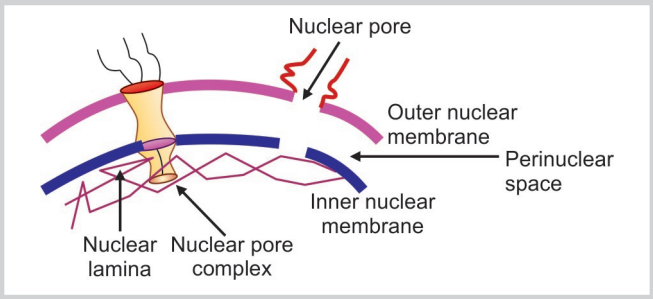


Fig. 1.17: Double layered nuclear membrane is perforated by multiple nuclear pores

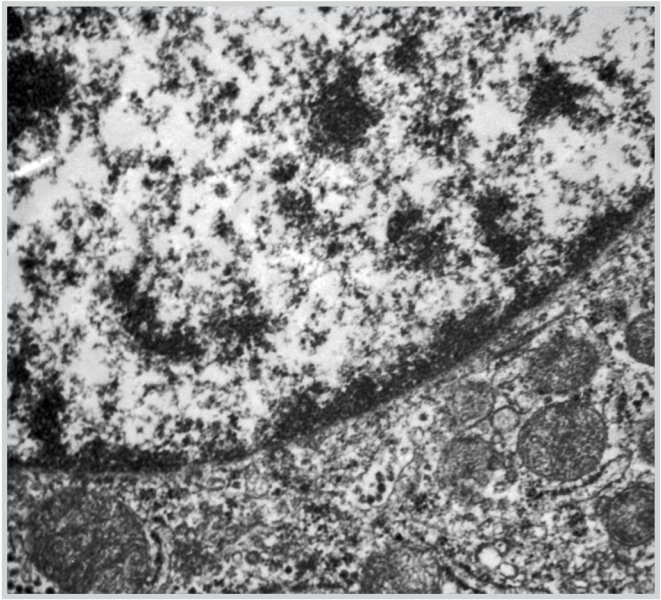


Fig. 1.18: Electron microscopic picture of nucleus and its membrane (*Courtesy:* Uma Nahar Saikia, Additional Professor, Department of Histopathology, PGIMER, Chandigarh, India)

Outer nuclear membrane is the outermost part of the nuclear membrane and is 6 nm thick. It is continuous with the endoplasmic reticulum. It is usually studded with multiple ribosomes on its cytoplasmic side that are involved in protein synthesis. The INM is parallel to ONM and is directly attached to the nuclear lamina. The space in between ONM and INM is known as perinuclear space. The width of this space is 50 nm. Both the ONM and INM are perforated by multiple nuclear pores.

The nuclear lamina is located in the nuclear side of the INM. It is made up of nuclear lamin that is intimately related to the cytoplasmic intermediate filaments. The lamins are broadly classified as either A or B type lamin depending on their amino acid sequence, behavior at mitosis and tissue specific patterns. Each lamin molecule consists of an N-terminal head, long "coiled-coil" α helical rod and globular C-terminal tail domain.[27] The central "coiled-coil" rod domains of two lamins interact to form lamin dimers and the lamin dimers are arranged with each other in an anti-parallel manner. The INM contains a number of integral proteins such as lamin B receptor (LBR), lamina associated polypeptide (LAP), MAN 1, emerin, nurim and ring finger binding protein (RFBP).[28]

Functions

- Acts as a physical barrier between the cytoplasm and nucleus.
- Various integral proteins such as LBR, LAP, RFBP help in chromatin remodeling and gene expression. LAP and lamin A/C bind with Rb-protein that further recruits histone deacetylases (HDAC), DNA methyl transferases (DNMT 1), histone methyl transferases (HMTase), and heterochromatin protein 1 (HP1). The action of these enzymes changes the higher-order conformation of chromatin and ultimately causes gene silencing by inhibiting transcriptional activation of E2F.[29,30]

Nuclear Pore

At certain positions, the ONM and INM fuse with each other and therefore, make the hole on the nuclear membrane. The nuclear pore is the direct communication site between the nucleus and cytoplasm. The diameter of each nuclear pore is about 100 nm. The number of nuclear pore varies from few hundreds to thousands depending on the metabolic activity of the cell. The nuclear pore complex (NPC) is the gateway of the nucleus across the double membrane nuclear envelope (**Fig. 1.17**).[31] The NPC selectively exchanges the macromolecules between the nucleus and cytoplasm.[30]

Nuclear pore complex consists of a cytoplasmic ring, a nuclear ring, and a distal ring connected by nuclear basket (**Fig. 1.17**).[32]

Function

The main function of the nuclear pore is the facilitation of the cytoplasmic to nuclear traffic and *vice versa*.

Nuclear Matrix

The nuclear matrix is the internal skeleton of the nucleus and consists of an RNA network, protein complexes, peripheral

BOX 1.14 Chromatin

- The uncoiled chromosome of the interphase nucleus
- Composed of:
  - DNA
  - Histone
  - Nonhistone proteins
- Types
  - Inactive heterochromatin
  - Active euchromatin
- Nucleosome:
  - The basic unit of chromatin
  - DNA encircles in two turns around a central octameric protein core containing two copies each of histone H2A, H2B, H3 and H4
  - Strings of nucleosomes are helically twisted and folded to form the higher order organization of chromatin

nuclear lamin, and residual nucleoli. The composition of the nuclear matrix is dynamic and varies with nuclear activities. The nuclear matrix protein (NMP) is tissue specific and participates in many vital cell functions, such as gene transcription and translation.

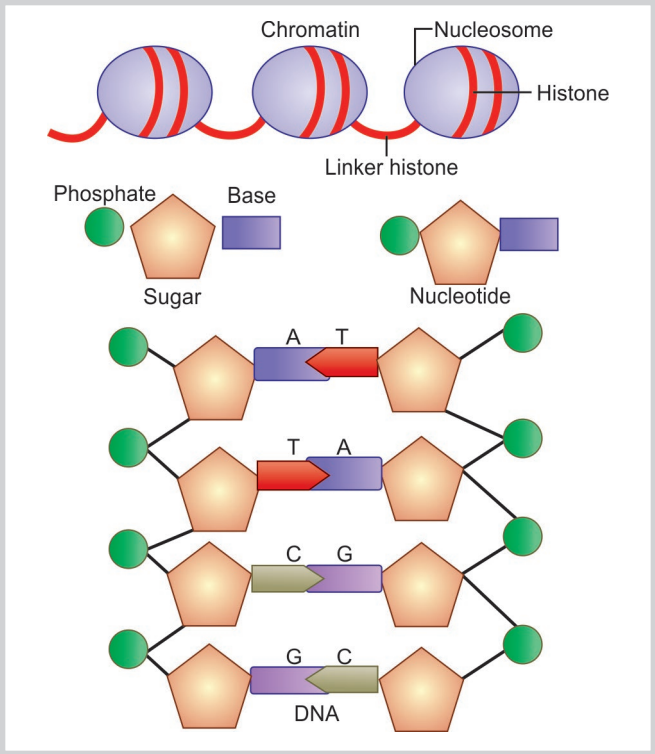


Fig. 1.19: Nuclear chromatin and DNA structure. Double helix DNA structure is made of sugar, phosphate back bone and four bases—adenine (A), guanine (G), cytosine (C) and thymine (T)

Nuclear Chromatin

Chromatin Structure

Chromatin represents the uncoiled chromosome of the interphase nucleus (**Box 1.14**). It is composed of DNA, histone, and nonhistone proteins.[33]

In the interphase nucleus, the individual chromosomes occupies specific position of the nucleus which is referred to as chromosomal territories and the chromosomes are separated by channels called interchromosomal domains. Chromatin can be classified as heterochromatin and euchromatin. The heterochromatin is the condensed portion of chromatin where genes are usually inactive. Heterochromatin usually is found on the nuclear membrane (**Fig. 1.2**) and can be further divided into facultative and constitutive. In case of facultative heterochromatin, the genes are inactive in certain cell types in certain stages of development. The constitutive heterochromatin consists of chromosome structural components such as telomeres and centromeres. Heterochromatin and euchromatin are seen only in ordinary light microscopy of fixed preparation of cells.

Nucleosome—Basic Unit of Chromatin

Nucleosome is the basic unit of chromatin. Each nucleosome consists of approximately 146 bp of DNA. This DNA encircles in two turns around a central octameric protein core containing two copies each of histone H2A, H2B, H3 and H4 (**Fig. 1.19**). The central core histone proteins are arranged as a (H3-H4)2 tetramer and two H2A-H2B dimers located on either side of the tetramer. Histone H1 is known as the linker histone. This linker histone binds to the DNA joining nucleosomes together and to core histone. The strings of linked nucleosomes are helically twisted into a 10 nm fiber, which, in turn, is folded into a 30 nm fiber and forms the higher order organization of chromatin. The thread of chromatin fiber makes a bend or loop and the DNA at the base of the loop is attached to the NMP. This is known as matrix attachment region (MAR). MAR takes essential role in gene expression.[34]

Nucleolus

Nucleolus is the subnuclear round to oval small structure within the nucleus and about 1 micron in diameter (**Box 1.15**). It is not a membrane-bound structure. Nucleolus is usually situated in the center of the nucleus; however, the position of the nucleolus may vary. The number of nucleolus may vary from 1 to 3. The size of the nucleolus depends upon the requirement of ribosome and protein synthesis. So, it is expected that metabolically active cell with higher amount of protein synthesis will have larger nucleoli. Nucleolus is easily detectable by light microscope. In hematoxylin and eosin stained histological section, the nucleoli are stained as deep eosinophilic round structures. In May Grunwald Giemsa stained cytology smears, the nucleoli are stained as light-blue colored structures.

BOX 1.15 Nucleolus

- Subnuclear, nonmembrane bound
- Round to oval small structure, about 1 micron in diameter
- Size of the nucleolus depends upon the requirement of ribosome and protein synthesis
- Contains: Protein and rRNA
- Formation: At the end of the mitosis around the tandemly repeated clusters of ribosomal DNA genes.
- Nucleolar organizing regions: Seen in homologues chromosomes of 13, 14, 15, 21 and 22.
- Functions:
  - The site of rRNA transcription, processing and ribosomal assembly.

The nucleoli are formed at the end of the mitosis around the tandemly repeated clusters of ribosomal DNA (rDNA) genes (**Fig. 1.20A**). These specific genetic loci of the origin of nucleoli are known as *nucleolar organizing regions (NORs).* The nucleolar organizer loci are seen in homologues chromosomes of 13, 14, 15, 21 and 22. Therefore at the end of mitosis, tiny 10 nucleoli appear to from the NOR of the five pair of chromosomes (total 10 chromosomes). These small nucleoli conglomerate to form a single larger nucleolus. The nucleolus contains protein and rRNA. The protein and rRNA are surrounded by chromosomal DNA of the nucleolus.

Structure

At the electron microscopic level, the nucleolus exhibits three major subregions[35] (**Fig. 1.20B**).

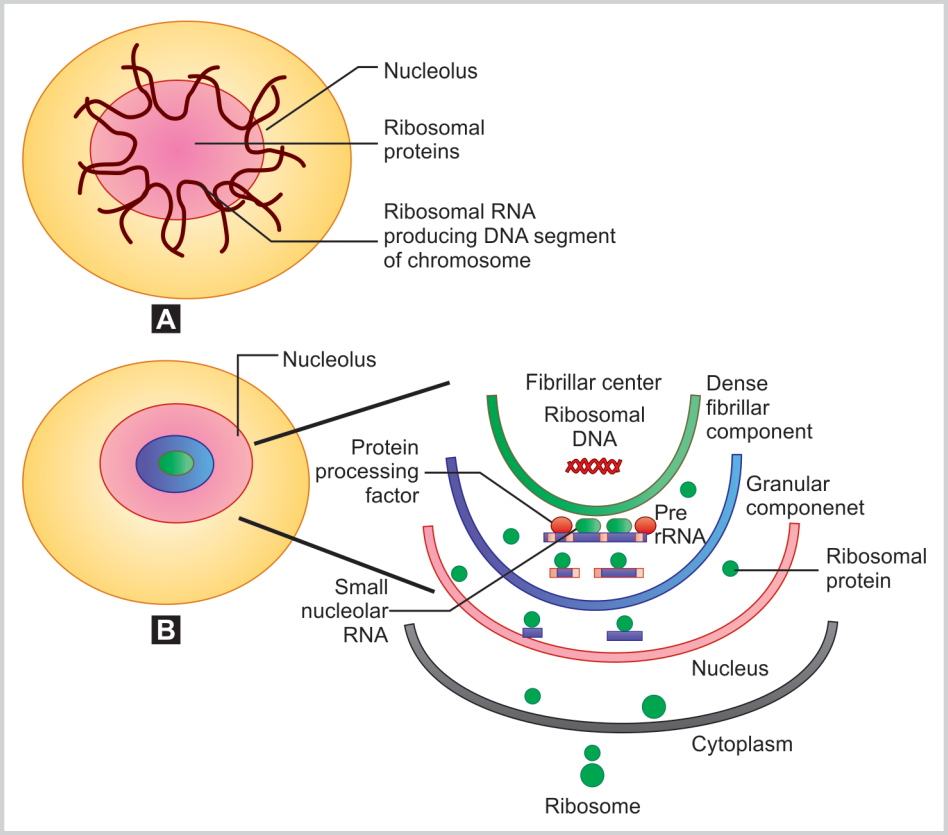
- Fibrillar center (FC): It looks like variable sized round structure with very low electron opacity.
- Dense fibrillar component (DFC): It is located on the outer rim of FC and is composed of densely packed fibrils.
- Granular components (GC): This is the outermost region and is composed of granules.

These different regions of nucleoli probably indicate the stages of RNA transcription and ribosomal assembly.

Function

The nucleolus is the site of rRNA transcription, processing, and ribosomal assembly.

- Ribosomal RNA transcription: Transcriptionally active rRNA genes are located in the FCs and DFCs. The transcription of rRNA genes occurs by RNA polymerase I enzyme. The primary transcript of the ribosomal DNA is the large 45S pre rRNA. This contains the 18S, 5.8S and 28S rRNAs. Subsequently, the preribosomal RNA transcripts are processed in the DFC with the help of small nucleolar RNA and other protein processing factors. A series of cleavages occur during the processing of preribosomal RNA to mature rRNA. In addition, considerable amount of methylation of the bases and ribose residues also happens. The various proteins in the NORs are selectively stained by the silver staining method and these proteins are labeled as AgNOR proteins.[36]
- Ribosomal synthesis (**Fig. 1.20B**): Outside the nucleus, the genes of the ribosomal proteins are transcribed and from the cytoplasm, these proteins are transported to the nucleolus. With the help of rRNA these ribosomal proteins are assembled within the nucleolus to form preribosome.



Figs 1.20A and B: A) Organization of nucleoli. Five pairs of chromosomal parts that produce ribosomal DNA with ribosomal proteins make nucleoli; B) Nucleolar structure and functions are highlighted

BOX 1.16 Deoxyribonucleic acid

- Double helical strands containing a sugar phosphate backbone and bases attached with the sugar molecule
- Sugar molecule: a pentose sugar attached with the phosphate by third and fifth carbon atom, alternatively
- Four base pairs: Adenine, Cytosine, Guanine and Thymine
- Adenine joins only with Thymine and Cytosine joins only with Guanine
- Nucleotides: The unit of pentose sugar, phosphate and nucleobase
- Gene: The specific portion of DNA with particular arrangement of nucleobases that carry the genetic information

The preribosome is transported back to the cytoplasm for final maturation.

DEOXYRIBONUCLEIC ACID

Deoxyribonucleic acid, the nucleic acid, carries the vital genetic information of the cell (**Box 1.16**). *The portion of DNA that carries the genetic information is known as gene*. Within the nucleus, DNA is coiled and supercoiled to make a thread-like structure known as chromosome. During cell division, the chromosomes are easily visible as distinct entity by light microscope. However, in the interphase, the chromosomes remain partly condensed and partly extended. Therefore, they are not visible as a distinct entity. There are 23 pairs of chromosomes, out of which 22 pairs are autosomes and one pair is sex chromosome. Sex chromosomes in the male are X and Y chromosomes, and in the female are X and X chromosomes.

Structure of Deoxyribonucleic Acid

Deoxyribonucleic acid is made of double helical strands containing a sugar phosphate backbone and bases attached with the sugar molecule.[37] Each strand of DNA is made up of alternate sugar and phosphate molecules. The sugar molecule is a pentose sugar and it is attached with the phosphate by 3rd and 5th carbon atom, alternatively. The nucleobase is attached with each sugar molecule and then links with the other base of the opposite strand by a weak hydrogen bond. There are four base pairs: adenine (A), cytosine (C), guanine (G) and thymine (T). Adenine and guanine are purine bases. Cytosine and thymine are pyrimidine bases. Adenine only joins with thymine and cytosine only joins with Guanine. This is known as complementary base pairing. There is another pyrimidine base known as uracil (U) that is found in RNA (**Fig. 1.19**).

Nucleotides

It is the basic structural unit of DNA. It consists of a pentose sugar, phosphate and nucleobase (adenine, cytosine, guanine or thymine). Nucleobase and sugar molecule form a nucleoside.

Gene

Gene is the specific portion of DNA with particular arrangement of nucleobases that carry the genetic information for making a particular protein. It is the sequence of A, T, C and G that determine the genetic information. Triplet of three consecutive bases of DNA is known as *codon* and each codon is specific for a particular amino acid. This specifies the sequence of amino acids, and subsequently, the protein formation. Only certain parts of the DNA are involved in carrying the genetic information, and the "in between part" of the DNA is commonly known as noncoding DNA or junk DNA. The exact biological function of the noncoding DNA is not known. However, evidences suggest that noncoding DNA interacts with micro-RNA and thereby controls transcriptional and translation of protein coding sequences.[38]

DNA Replication

Deoxyribonucleic acid replication is a semi-conservative process by which genetic inheritance is maintained. When a cell divides, DNA replication process happens. Here each strand of DNA serves as a template and an identical complementary daughter DNA strand is formed. Each of the newly formed daughter cell contains DNA make up of one original strand and one freshly made strand. Therefore, the DNA double helix replication is a semi-conservative process. This replication process is well controlled and free of mistakes because of stringent proof reading and error checking mechanisms (**Fig. 1.21**).

Deoxyribonucleic acid replication process needs following enzymes:

- DNA polymerases: These are the major enzymes in DNA replication process. These enzymes help in the polymerization

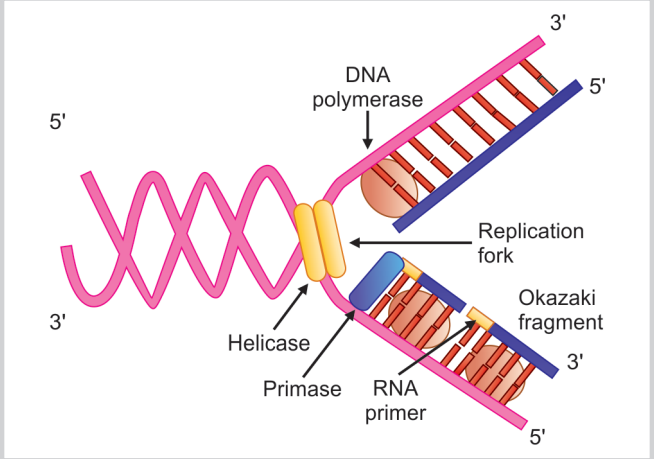


Fig. 1.21: DNA replication steps are highlighted in this schematic diagram. Helicase enzyme breaks the double stranded DNA and a replication fork is formed. With the help of DNA polymerase enzyme DNA strand is made. In the leading strand DNA is synthesized in continuous manner at the direction of replication fork whereas in lagging strand DNA is synthesized in opposite direction as small segments. These small segments are known as Okazaki fragments

of deoxyribonucleotides into the DNA strand. The DNA polymerase enzyme reads the intact template DNA strand to make the complementary DNA strand. On the basis of sequence homology and structural similarities, DNA polymerases are classified into five major families:[39] A, B, C, X and Y. The three major varieties of eukaryotic DNA polymerases α, δ and ε belong to family B. The mitochondrial DNA polymerase γ belongs to family A. There are two important and *fundamental properties of DNA polymerases*:

- They only can add free nucleotides in the 5′ to 3′ direction.
- They need a preformed primer strand which is attached to the template DNA by hydrogen bonding. DNA polymerase adds a deoxyribonucleoside 5′ triphosphate to the 3′ OH group of the primers strand.

DNA replication is the coordinated activities of various enzymatic processes. The basic mechanisms of DNA replication process is evolutionary preserved. DNA replication events are initiated in many hundreds of points in chromosomes. This initiation point of the segment of DNA is known as *origin*. The protein complex that acts on the origin as initiator of the DNA replication is known as origin recognition complex (ORC). The human ORC is site nonspecific. However, it is suggested that in somatic differentiated cells human ORC binds to genomic DNA with certain specificity.[40] DNA is replicated in S phase of the cell cycle. Total time of replication of DNA is usually fixed.

Steps of DNA Replication[41] (Box 1.17)

For the purpose of description, the replication process can be divided into series of steps:

1. At first two strands of DNA are separated at a particular point known as origin. Here, the initiator protein along with other associated protein forms a prereplication complex that separates the two strands of DNA. Therefore, a fork-like structure is formed known as *replication fork*. Helicases enzymes break the hydrogen bonds in between the bases and the unwound DNA strands are stabilized by single stranded DNA binding proteins.
2. The binding of RNA primase in the initiation point of the 3′ to 5′ parent chain: there is extension of the RNA primers by DNA polymerase that binds to the DNA nucleotides of the 3′ to 5′ strand due to the hydrogen bonds between the bases.
3. DNA polymerase adds the matching loose nucleotide: DNA polymerase can act only from 5′ to 3′ direction. Therefore, DNA replication is different in two strands of DNA. Original 5′ to 3′ strand of DNA replication starts from 3′ end and proceeds to the direction of the breakage of the replication fork. The strand of DNA here is known as *leading strand*. In leading strand DNA is synthesized in continuous manner. In other strand of DNA, known as *lagging strand*, the process of DNA synthesis is in discontinuous manner, opposite to the direction of the replication fork. This occurs in the multiple areas of the DNA strand. Therefore, the multiple small pieces of DNA are synthesized that are known as *Okazaki fragments*.
4. Joining of intact lagging strand: The RNA strands are removed by the action of *RNase enzyme* and *DNA Pol I exonuclease*. The lagging strands are joined by DNA ligase.

Ultimately, the DNA replication is terminated. Each double helix DNA contains one old template strand and one newly synthesized fresh strand.

BOX 1.17 DNA replication

- Semiconservative process
- DNA replication needs:
  - DNA template
  - Helicase enzymes
  - DNA polymerases and other associated factors
- Steps
  - The initiator protein along with other associated protein forms a prereplication complex
  - Helicases enzymes break the hydrogen bonds in between the bases
  - Replication fork is formed
  - Extension of the RNA primers by DNA polymerase
  - DNA polymerase adds the matching loose nucleotide
  - In leading strand, DNA is synthesized in continuous manner from 3′ to 5′ direction
  - In lagging strand, the process of DNA synthesis occurs in discontinuous manner opposite the direction of the replication fork
  - Okazaki fragments: The multiple small pieces of DNA in lagging strand
  - The lagging strands are joined by DNA ligase

DNA Transcription and Protein Synthesis

The principal key factor of protein synthesis remains in the DNA sequence of the nucleus. At first, the portion of DNA template is copied into the messenger RNA (mRNA). This mRNA is processed within the nucleus, and finally comes out from the nucleus to the cytoplasm through nuclear pore. Within the cytoplasm, on the small subunit of ribosome attached with RER, the mRNA is decoded and protein is synthesized with the help of t-RNA (**Box 1.18**).

BOX 1.18 DNA transcription

- The information of DNA is transferred to the corresponding mRNA
- The mRNA synthesis needs:
  - A DNA chain
  - Transcription factors
  - RNA polymerase II
- TATA binding protein (TBP) binds with TATA box of DNA
- The transcription factors complex help in binding RNA polymerase to DNA
- Helicases enzyme of TFIIH unwinds DNA
- RNA polymerase now moves from the promoter and synthesis of mRNA from the DNA strand starts
- The terminal codon of DNA stops the m-RNA synthesis by RNA polymerase

The two major steps of the protein synthesis are:

- Transcription: It is the process of making mRNA (messenger RNA) from DNA.
- Translation: The process of synthesizing protein from the mRNA code is known as translation.

Transcription

In this process, mRNA is formed from the particular sequence of nucleotides of DNA carrying the genetic information to make a particular protein. Therefore, in this step, the information of DNA is transferred to the corresponding mRNA. The basic information of DNA remains same, so the process is known as transcription (**Fig. 1.22**).RNA is essentially same as DNA in structure except in certain points:

- This is a liner polymer of nucleotides
- The sugar moiety is ribose
- RNA contains the base uracil (U) instead of thymine (T)

There are three important steps of transcription: initiation, elongation, and termination.

- Initiation: Initiation of the mRNA synthesis needs a DNA chain, transcription factors and RNA polymerase II. At first TATA binding protein (TBP) binds with TATA box of DNA. TBP is a part of general transcription factor called TFIID. The binding of TFIID promotes the binding of another protein known as TFIIB. This complex helps in binding RNA polymerase, to DNA. After the recruitment of RNA polymerase another two transcription factors, TFIIE and TFIIH binds, with this complex and initiation complex is completed.
- Elongation: The helicases enzyme of TFIIH unwind DNA, and the RNA polymerase starts synthesis of mRNA from the DNA strand. RNA polymerase now moves from the promoter and elongation phase starts. The DNA strand is read from 3′ to 5′ direction and subsequently RNA strand is made from 5′ to 3′ direction.
- Termination: When the RNA polymerase reaches to the terminal codon of DNA, the mRNA synthesis is stopped.

Translation

In translation phase, information of mRNA is decoded and the protein is synthesized. The newly formed mRNA passes through the nuclear pore and binds properly with the small unit of ribosome. Ribosome contains many proteins and rRNA. There are four nucleotides (adenine, guanine, cytosine and uracil) in RNA and each group of three consecutive nucleotides of mRNA is called a *codon* (**Box 1.19**). Each codon indicates one specific amino acid. More than one codon may also specify a particular amino acid. Now for each codon of m-RNA there is a specific transfer RNA (tRNA) carrying the complementary codon nucleotide sequence called *anticodon*. Each such tRNA molecule is linked with a particular amino acid. The recognition and attachment of the specific amino acid with this tRNA is dependent on enzymes known as aminoacyl-tRNA synthetase.

At first, mRNA is attached with the surface of ribosome. Then specific tRNA is attached to the start codon of the mRNA carrying a particular amino acid. Ribosome moves from 5′ to 3′ direction of mRNA and then a new tRNA with another particular amino acid is attached next to the previous one. The former tRNA is released from the mRNA. This process continues till the end of the tRNA meets the stop codon and ribosome stops translation. The complete protein is synthesized and comes to the cytoplasm (**Fig. 1.23**).

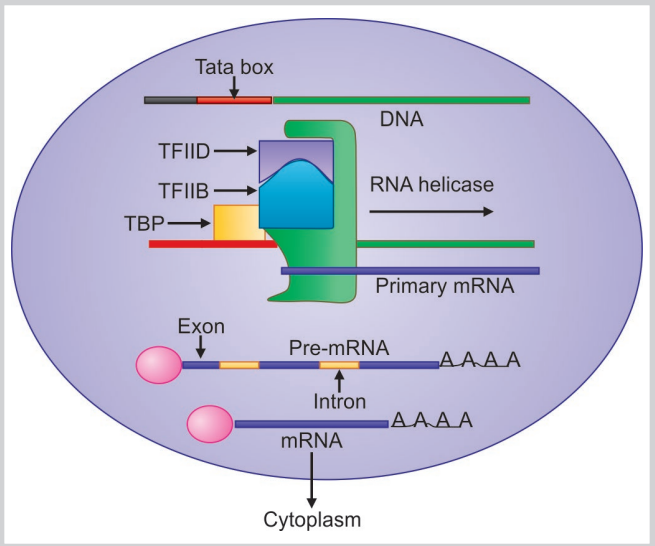


Fig. 1.22: Schematic diagram showing mRNA transcription from DNA. Intron regions are sliced out and ultimately mRNA is formed from pre-mRNA

BOX 1.19 Translation of mRNA code

- Information in mRNA is decoded to synthesize protein
- Codon:
  - A group of three consecutive nucleotides of mRNA
  - Each codon indicates one specific amino acid
- Anticodon:
  - A specific tRNA carrying the complementary codon nucleotide sequence
  - Each tRNA molecule is linked with a particular amino acid.
- Steps
  - mRNA is attached with the surface of ribosome
  - The specific tRNA with amino acid is attached with the start codon of the mRNA
  - Ribosome moves from 5′ to 3′ direction of mRNA and then a new tRNA with another particular amino acid is attached
  - A complete chain of amino acid of protein is formed

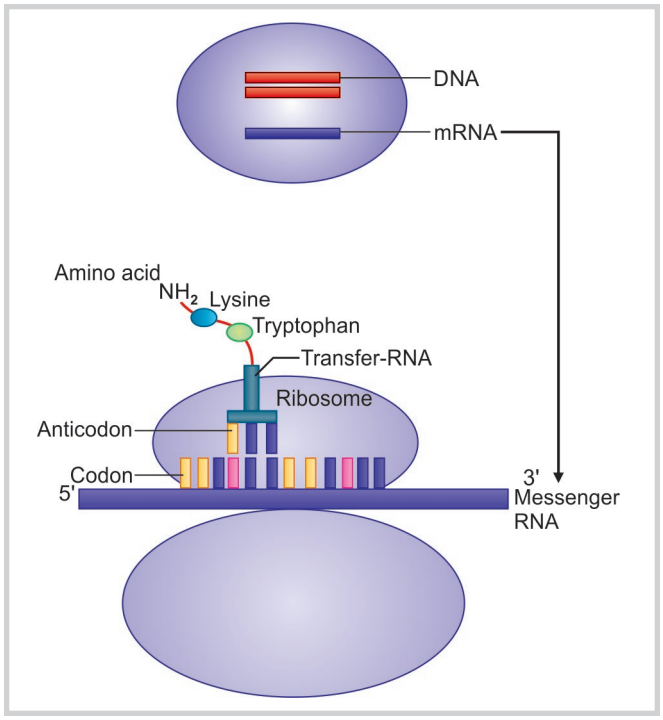


Fig. 1.23: Translation of mRNA to protein synthesis in ribosomal surface is highlighted. Information of mRNA is decoded and tRNA with complementary anticodon brings a specific amino acid to form a protein

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CHAPTER 2

Cell Cycle and Cell Proliferation

Chapter Contents

The process of dividing a single cell into two or more daughter cells is known as cell division.

CELL CYCLE

Somatic cell is divided into two daughter cells by mitosis. The cell continuously goes through mitosis and the preparatory phase before mitosis. This preparatory phase is also known as interphase, which is the combination of resting phase and deoxyribonucleic acid (DNA) synthesizing phase. The interphase includes G_0, G_1, S, and G_2 phase.

Eukaryotic cell cycle is divided into four main phases: G_1, S, G_2, and M phase (**Fig. 2.1**).

Synthetic or S phase: In this phase, DNA is synthesized and chromosome is duplicated. The time taken for S phase is usually 10–12 hours and this phase occupies half the time of cell cycle. S phase contains variable amount of DNA from 2n to 4n (here n denotes the haploid DNA content).

Mitotic or M phase: After the completion of chromosomal duplication, the chromosomes segregate and the cell enters into the mitotic phase. Here each cell divides and the chromosomal material is divided equally in these two daughter cells. This phase contains 4n DNA. When the daughter cells are generated, each one contains 2n DNA.

Gap 2 or G_2 phase: The dividing cell takes a time gap to grow and prepare for mitosis after the completion of S phase. This is known as G_2 phase.

Gap 1 or G_1 phase: After the completion of the mitotic phase, the cell takes some time to grow before undergoing S phase. This is

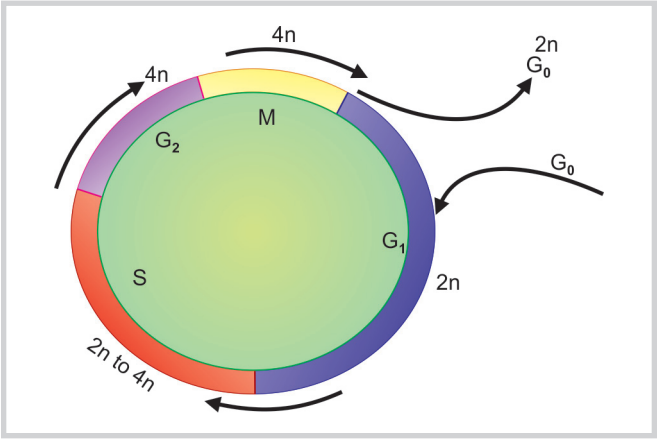

Fig. 2.1: Schematic diagram of cell cycle. The cell in cell cycle passes through G_1, S, G_2, and M phase. G_0 is the resting phase of the cell

BOX 2.1 Cell cycle

G_0 phase:
- The cell does not divide and does not undergo cell cycle
- Diploid (2n) DNA (here n denotes the haploid DNA content)

Gap 1 or G_1 phase:
- This is in between the mitotic and synthetic phase
- Diploid (2n) DNA content

Synthetic or S phase:
- It contains variable amount of DNA from 2n to 4n
- DNA is synthesized

Gap 2 or G_2 phase:
- This is in between the S and M phase
- The dividing cell takes a time gap to grow and prepare for mitosis after the completion of S phase
- The cell contains tetraploid (4n) DNA

Mitotic or M phase:
- Each cell divides and the chromosomal material is divided equally in these two daughter cells
- The cell contains tetraploid (4n) DNA

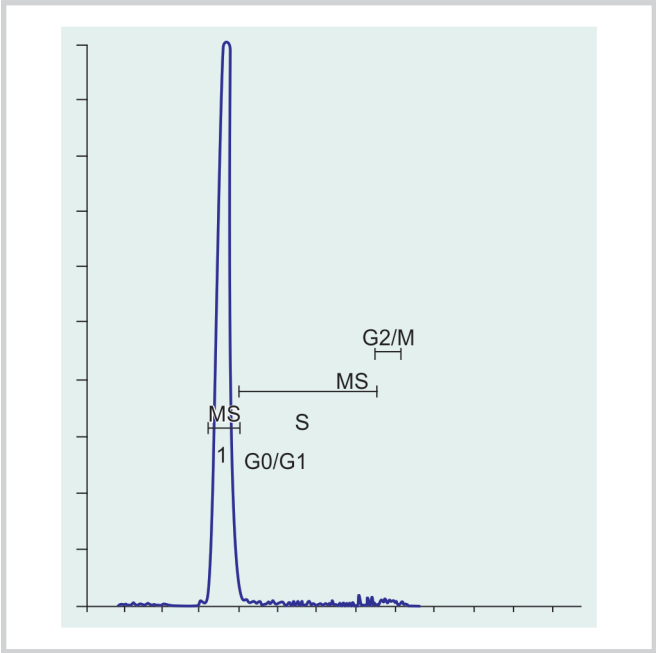

Fig. 2.2: Deoxyribonucleic acid flow cytometry shows cells in different phases of cell cycle

known as G_1 phase. In this period, the cell grows and generates more cellular organelles. The total amount of DNA is diploid (2n). The cell remains in this phase till it enters to S phase for the next round of cell division.

There is another phase of cell cycle when a cell does not divide and does not undergo cell cycle. This phase is known as G_0 phase. The cell may remain in this phase for days to years till it receives stimulation for cell division (**Box 2.1**).

Duration of each phase of cell cycle varies widely. However, the major bulk of cell cycle time is taken in G_1 and S phase. In 24-hour-duration of cell cycle, G_1 and S phase take near about 19 hours, whereas M phase takes only 1 hour time. Depending on the period of staying in G_0 phase, the cells may be divided into three types:

Labile cell: The cells of this type remain short period in G_0 phase and quickly enter into the cell cycle. The labile cells are rapidly proliferating population of cells. Intestinal epithelial cells are the example of labile cells.

Stable cells: This type of cells remains in G_0 phase for long time until there is stimulation to divide. Liver cells, lining of kidney tubular cells, and pancreatic cells are stable cells. They usually do not divide until a fraction of them are destroyed by injury or inflammation.

Permanent cell: Some cells remain permanently in G_0 phase and never undergo cell division, such as neuronal cell. These cells are known as permanent cell.

Deoxyribonucleic Acid Content and Cell Cycle

A human somatic cell contains 46 chromosomes (2n). Here "n" is designated as haploid DNA content of the genome. The cell in G_0 and G_1 phase of the cell cycle contains 2n amount of DNA. The cell in S phase synthesizes DNA and therefore contains variable amount of DNA from 2n to 4n. M phase contains 4n DNA and each daughter cell contains 2n DNA. Cell cycle can be analyzed by flow cytometry (FCM) or image ploidy analysis and the relative content of DNA is measured by these techniques (**Fig. 2.2**).

Cell Division

There are two types of cell division: Mitosis and meiosis.

Mitosis

Here, the parent cell is divided into two identical daughter cells with identical chromosomal material in each nucleus. **Figure 2.3** shows typical mitotic figure in cytology smear. There are five stages of mitosis (**Box 2.2**): prophase, prometaphase, metaphase, anaphase, and telophase (**Fig. 2.4**).

Prophase

It is the preparatory phase of mitosis proper. The following changes are seen:
- The nucleoli and nuclear membrane both disappear
- The duplicated chromosomes in G_2 phase condense and form two identical chromatids. These sister chromatids are joined together in centromeres by a special protein known as cohesin. Centromere is a DNA sequence to which proteins are attached to form a kinetochore, the complex protein structure
- The pair of original centrioles replicate and two pair of centrioles are formed that travel to the opposite poles
- The microtubules start to polymerize to generate mitotic spindle.

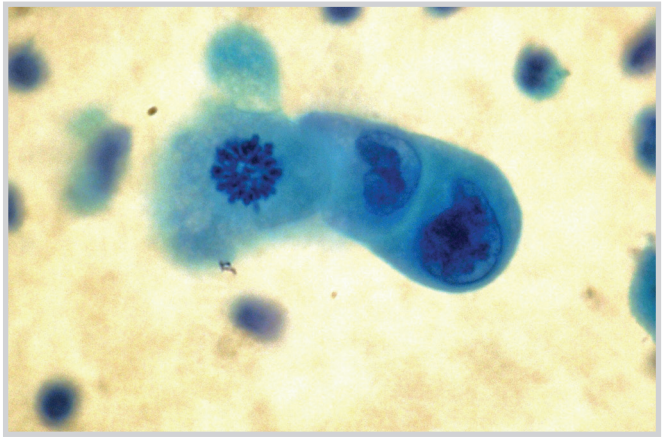

Fig. 2.3: Mitotic figure in cytology smear (Papanicolaou's stain X OI)

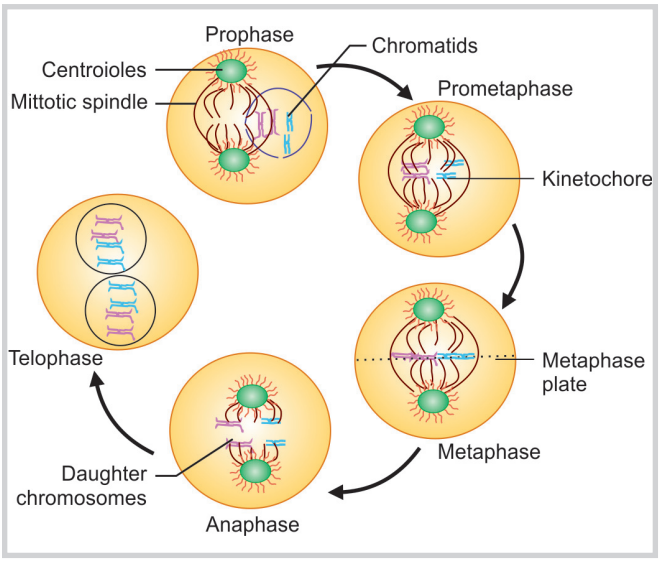

Fig. 2.4: Schematic diagram of different phases of mitosis

BOX 2.2 **Mitosis**

Prophase
- The nucleoli and nuclear membrane both disappear
- The chromosomal pair condenses and forms two identical chromatids
- The centrioles replicate and two pair of centrioles form that travel to the opposite poles
- Microtubules polymerize and the mitotic spindle is formed

Prometaphase
- Mitotic spindle formation completes
- The kinetochore of each sister chromatids attaches with the microtubules of the mitotic spindle

Metaphase
All the chromatids are well-aligned to the equatorial plane of the mitotic spindle

Anaphase
- The two sister chromatids are separated
- The chromatids move toward the opposite pole and two identical set of chromosomes reach to each pole

Telophase
- The remnants of the mitotic spindle disappear
- The nuclear membrane and new nucleoli are formed

Prometaphase

This is the transition phase between prophase and metaphase. In this phase, microtubules complete the formation of the mitotic spindle. Some microtubules are extended from the pole to the equatorial plane and others are extended from one pole to the other pole. Here, the kinetochore of each sister chromatids attaches with the microtubules of the mitotic spindle. Each kinetochore attaches with the microtubules called kinetochore microtubules that emanates from the opposite pole of the spindle.

Metaphase

In metaphase, all the chromatids are well aligned to the equatorial plane of the mitotic spindle and the journey of the chromatids toward the each pole is set. Every kinetochore should be properly attached to the kinetochore microtubule of the mitotic spindle. This is very essential for the proper distribution of the chromosome to the daughter cell and to avoid aneuploidy. Any unattached kinetochore generates signal to halt the mitosis in this phase, and this is known as mitotic spindle checkpoint.

Anaphase

Here at first, the two sister chromatids are separated and become separate daughter chromosomes. Now the kinetochore microtubules shorten and spindle tubules elongate. The two poles go apart and simultaneously pull the chromosomes pair toward the pole. The pair of each chromosomes move to the opposite pole at the same speed.

Telophase

This is just the reversal of prophase and metaphase. Here, the remnants of the mitotic spindle disappear; nuclear membrane and new nucleoli are formed.

Cytokinesis

In the late anaphase, the process of cytokinesis starts and a constriction of cytoplasm develops in the equatorial plane of the original mitotic spindle. The process of cytokinesis continues till the end of the telophase.

Meiosis

Meiosis occurs in the gamete producing cells. In this process of cell division, the single diploid cell divides into four daughter

cells, each contains half of the parental chromosomes (**Fig. 2.5**). The cells with haploid chromosome need to maintain the diploid number of chromosomes in the fertilized cell. Meiosis occurs in the process of gametogenesis to produce sperm or ovum (**Box 2.3**).

Meiosis is divided into two parts: (1) First meiotic division (Meiosis I or reductional division) and (2) second meiotic division (Meiosis II).

The first meiotic division is known as reductional division because in this phase, each member of the homologous chromosome goes to one each cell and each one daughter cell contains haploid chromosome. During the S phase prior to first meiotic I, the DNA replicates and each chromosome produces two identical sister chromatids. The nonsister chromatids exchange their genetic material that is known as *crossover* (**Box 2.4**). Unlike mitosis, both the sister chromatids of the homologous chromosome go to one each nucleus. The meiosis I is subsequently followed by the second meiotic division, meiosis II that is mechanically same as mitosis. After the end of the meiosis II, a total of four haploid daughter cells are produced.

Meiosis I: Prophase of the meiosis is divided into five phases: leptotene, zygotene, pachytene, diplotene and diakinesis.

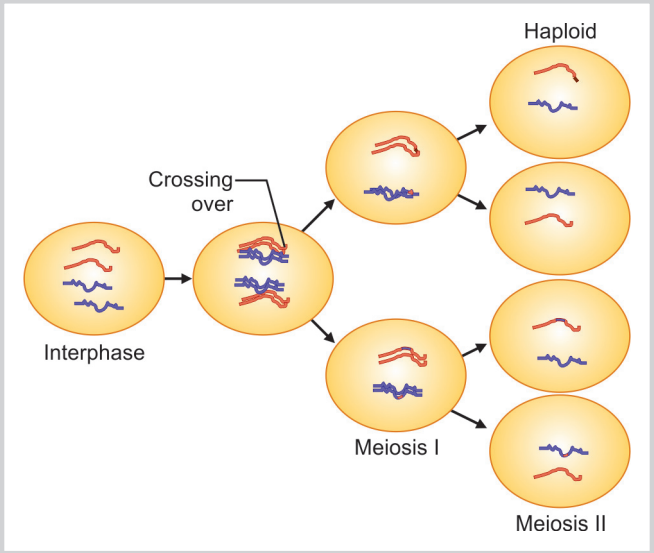

Fig. 2.5: Schematic diagram of different phases of meiosis

BOX 2.3 Meiosis

Meiosis I or reductional division: Each member of the homologous chromosome goes to one each cell

Meiosis I:
- Prophase of the meiosis is divided into five phases: leptotene, zygotene, pachytene, diplotene, and diakinesis
- Synapsis occurs in zygotene phase
- Crossover occurs in pachytene phase

Meiosis II: Mechanically same as mitosis

BOX 2.4 Crossover

- Overlapping of homologous region of the matching chromosome of nonsister chromatids in pachytene phase of prophase I
- The double-stranded DNA breaks into single strand
- In the crossover region, DNA breaks in both the nonsister chromatids
- Exchange of genetic information occurs in crossover region
- Paternal DNA exchanges information with maternal DNA
- Maintains genetic diversity

Leptotene: Progressive condensation and coiling of the chromosome occurs in this phase. The chromosome looks like long convoluted thread.

Zygotene: In this phase, both the homologous chromosomes are closely associated and aligned completely with each other point by point in their entire length. This is known as synapsis. This paired homologous chromosome in synapsis is known as bivalent chromosome.

Pachytene: In this phase, the chromosomes become more condensed and thick. The homologous chromosomes begin to move away but in certain regions they are overlapping like a cross. In those overlapping zones of nonsister chromatids of the homologous chromosome, exchange of genetic information takes place (**Fig. 2.6**). This is known as *crossover* (chiasmata). This is a very important event as exchange of genetic information occurs in crossover region and this gives rise to genetic heterogeneity in the gametes.

Diplotene: In this phase, homologous chromosome separates completely except in the points of crossover regions.

Diakinesis: This is the end stage of the prophase I where the chromosome condenses fully and the four tetrad chromatids are visible. Mitotic spindle begins to form now. The nuclear membrane and nucleoli dissolve.

Metaphase I: In this phase, the homologous chromosome with two sister chromatids attach to the kinetochore microtubules and align to the equatorial plane of the mitotic spindle.

Anaphase I: The kinetochore microtubules begin to contract and the whole homologous chromosome with sister chromatids move to one pole of the mitotic spindle. Unlike mitosis there is no such disjunction of the sister chromatids in meiosis and the centromeres remain intact.

Telophase I: The chromosomes reach to the each pole and each daughter cell receives haploid number of chromosome. Mitotic spindle dissolves, nuclear membrane and nucleoli appear and cytokinesis starts.

Meiosis II

It is mechanically similar to mitosis and can be divided into prophase II, metaphase II, anaphase II and telophase II. In the anaphase II, there is disjunction of the sister chromatids and

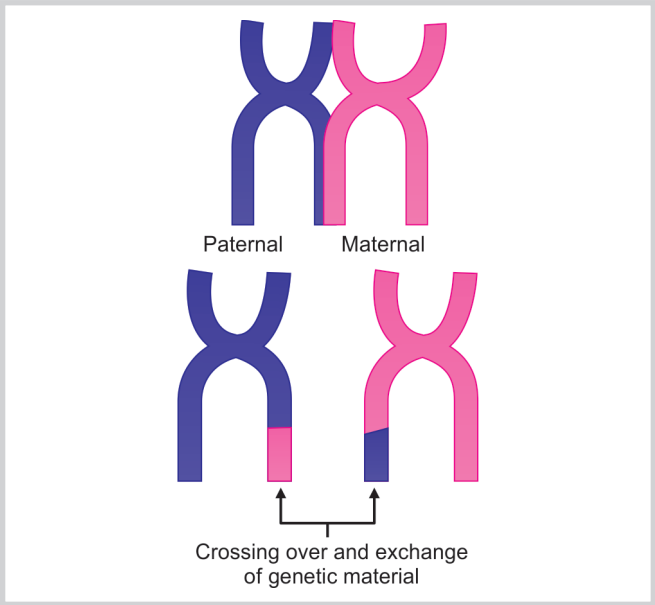

Fig. 2.6: Schematic diagram of crossing over with exchange of genetic material. Genetic diversity is maintained with the help of crossing over

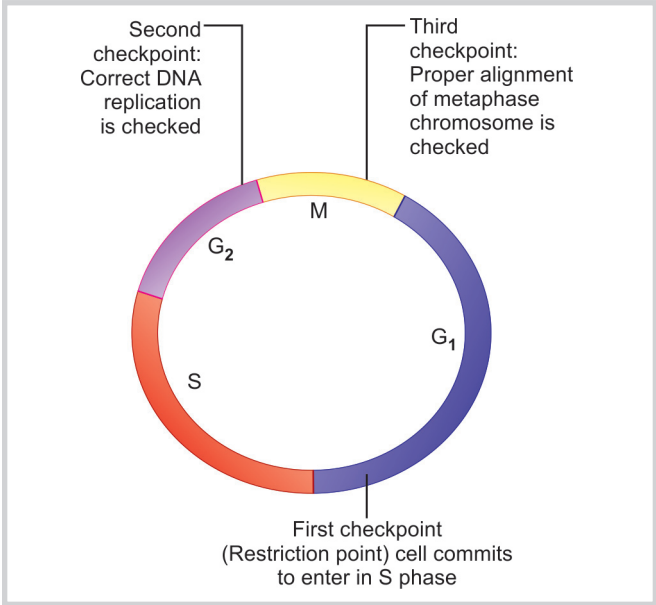

Fig. 2.7: Three different points of cell cycle restriction is highlighted in this schematic diagram

breakage of centromeres. The sister chromatids, i.e. chromosome, move towards the opposite pole.

Meiosis occurs in ovary and testis to produce gamete. Each gamete contains haploid chromosome. During fertilization of ovum with sperm, a complete diploid cell is generated from the fusion of the two haploid cells. Sperms contain either 23Y or 23X chromosome and ovum has 23X chromosome. Fertilization of ovum by the sperm containing 23Y generates male embryo whereas fertilization of the ovum by the sperm containing 23X generates female embryo.

Cell Cycle: Checkpoint

Control of the cell cycle is very important for the growth and development. For the last billion years, the mechanism of cell cycle control is almost the same. The important features of cell cycle control system are:

- It is very robust
- It is adaptive
- The control switches or restriction points are binary off and on that means once initiated they are usually irreversible[1].

The control points or cell cycle restrict points are located (**Fig. 2.7**):

- *Near the end-point of G_1 phase of the cell cycle*: The control point near the end of G_1 phase of the cell cycle is known as restriction point. Here, the environment is assessed and if the surrounding environment is favorable then the cell is allowed to enter into the S phase of the cycle.
- *At the junction of G_2 and M phase*: In the G_2/M checkpoint two fundamental factors are assessed: whether all DNA properly replicated or not, and the environment is favorable for proliferation or not?
- *In the M phase at the junction of metaphase and anaphase*: If all the chromosomes are properly aligned and attached to the kinetochore microtubules in the equatorial region of the mitotic spindle then the cell is allowed to go to metaphase to anaphase. Therefore, this checkpoint is also known as spindle assembly checkpoint.

Cell Cycle Regulator Proteins

The journey through the cell cycle checkpoint requires the activity of family of protein kinase enzymes known as cyclin-dependent kinases (CDKs).[2] These enzymes are structurally related to each other and are activated when they combine with another group of proteins known as cyclin (**Box 2.5**). A specific CDK can interact with a number of different cyclins, and a cyclin can interact with different CDKs.[3]

Activated CDKs play a key role in cell cycle control by *phosphorylation* of various protein enzymes essential for activating the genes involved in DNA synthesis and replication (**Fig. 2.8**). They also control nutrient uptake, breakdown of nuclear membrane, assembly of mitotic spindle and chromosomal condensation. The level of CDKs in the cell is constant; however, the cyclin level oscillates in different phases of cell cycle. The increased amount of cyclin-CDK complex (activated CDKs) triggers various cell cycle events. Depending on the binding of the CDKs in different stages of the cell cycle, cyclins are classified in three major classes:

1. Cyclins acting on G_1/S transition (G_1/S cyclin): Cyclin D and E
2. Cyclins acting on S phase (S cyclin): Cyclin A, cyclin E
3. Cyclins acting on M phase (M cyclin): Cyclin B and Cyclin A.

In a resting cell that comes out from the mitotic phase, retinoblastoma (Rb) protein is tightly bound with E2F transcription

BOX 2.5 Cyclin-dependent kinases

- CDK is activated by binding with specific cyclin
- Activated CDK phosphorylates the enzymatic proteins
- Phosphorylated active enzymes act on specific genes involved in DNA synthesis and replication
- CDK level is constant in a cell
- Cyclin level oscillates in different phase of cell cycle

Abbreviations: CDKs, cyclin-dependent kinases; DNA, deoxyribonucleic acid.

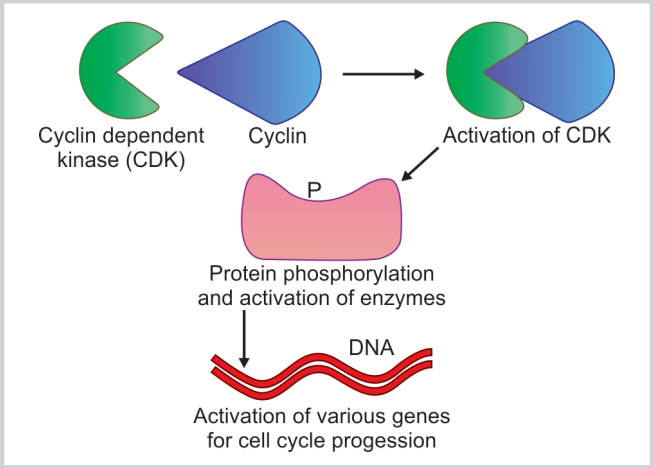

Fig. 2.8: Cyclin-dependent kinase is activated by combining with cyclin. Activated cyclin-dependent kinase causes phosphorylation and subsequently activation of various other cell cycle enzymes

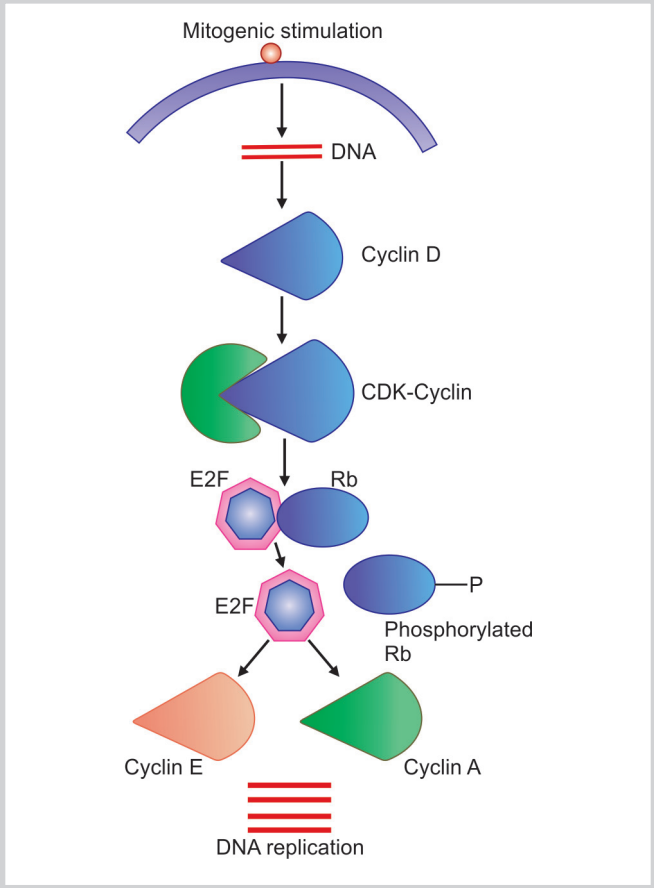

Fig. 2.9: External mitogenic stimulation activates gene to produce cyclin D. Cyclin-cyclin-dependent kinase complex phosphorylates retinoblastoma protein and liberates E2F, the transcription factor. This E2F stimulates deoxyribonucleic acid to produce various cell cycle enzymes such as cyclin E and A that help in deoxyribonucleic acid replication

factors. The cells cannot go into the proliferation phase due to the unavailability of E2Fs (**Fig. 2.9**). The stimulation of the cell by external mitogenic factors increases the level of cyclin D, which combines with respective CDKs (CDK4 and CDK6). The activated CDKs then phosphorylate Rb protein and release E2F transcription factors. Released E2F stimulates cyclin E and cyclin A and other large set of factors essential for DNA replication. Cyclin E-CDK complex can also phosphorylate Rb protein by itself and, therefore, behaves as a positive feedback loop to inactivate Rb protein and S phase entry of the cell. Therefore, phosphorylation of Rb protein by activated CDKs plays an important role in cell cycle initiation.[4]

M cyclin (Cyclin B) functions in M phase of the cell cycle by activating CDK1. Cyclin B-CDK1 complex controls the entry of the cell in mitotic phase by controlling G^2-M checkpoint. It phosphorylates the various proteins leading to the breakdown of the nuclear envelope, formation of mitotic spindle, and attachment of the chromatids with the spindle.

The specific cyclin-CDK complex acts mainly in the G_1/S and G_2/M checkpoint by phosphorylation of proteins. However, the metaphase to anaphase transition is regulated by protein destruction. Anaphase promoting complex (APC) is activated by the Cdc20. This activated APC catalyzes the ubiquitylation and destruction of securing protein[5] (**Fig. 2.10**). This securing protein is responsible for holding the two sister chromatids. Therefore, destruction of securing protein separates the sister chromatids and promotes the cell cycle progression from metaphase to anaphase. APC/C also destroys S and M cyclins and therefore inactivates CDKs within the cell (**Fig. 2.10**).

Inhibition of Activated Cyclin-dependent Kinases[2]

Active CDKs can be inhibited by: (1) the phosphorylation at two sites near the amino terminus (threonine14, tyrosine15), (2) by CDK inhibitory proteins (cyclin-dependent kinase *inhibitors*), such as p21, p27, p16 and p15, and (3) removal of cyclin from CDK-cyclin complex.

CELL CYCLE CONTROL AND CANCER

Uncontrolled cell proliferation due to deregulation of cell cycle engine is responsible for cancer. One or more cell cycle checkpoints are disrupted in cancer[6] (**Box 2.6**).

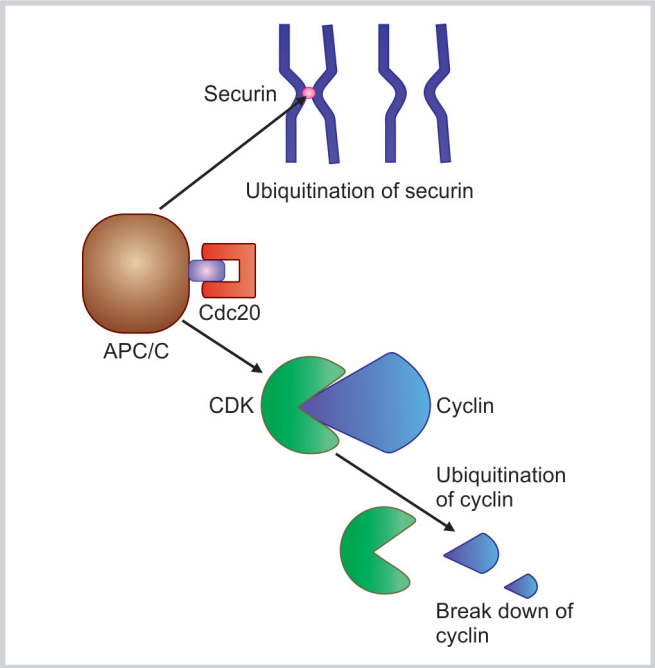

Fig. 2.10: Schematic diagram shows the mechanisms to control metaphase anaphase transition

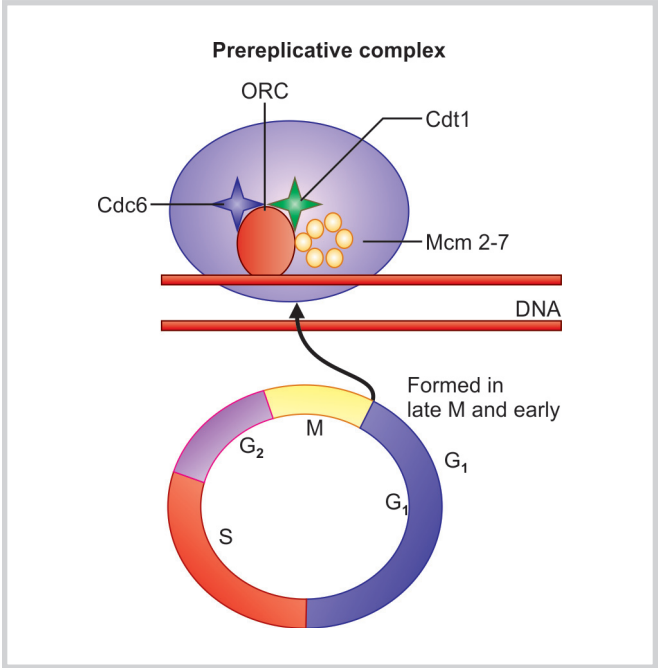

Fig. 2.11: Schematic diagram shows prereplicative complex formation by origin recognition complex, cell division cycle 6, Cdt1 and mini-chromosome maintenance 2–7

BOX 2.6 **Cell cycle checkpoint and cancer**

- G_1 entry checkpoint and cancer:
  - Retinoblastoma gene inactivation due to mutation or hypermethylation
  - Cyclin D1 and CDK4 gene amplification
  - HER-2 gene amplification
- DNA replication license and cancer:
  - Deregulation of the prereplicative complex system
- DNA damage, repair and cancer:
  - The defect in DNA damage checkpoint of the cell cycle: Mutation of ATM substrate modulators such as mediator of DNA damage checkpoint 1, p53 binding protein 1 and BRCA1

Abbreviations: DNA, deoxyribonucleic acid; ATM, *ataxia-telangiectasia-mutated; CDKs,* cyclin-dependent kinases

Entrance to G_1 checkpoint and cancer: "Mitogenic stimulation-active G_1-CDK-pRB phosphorylation-active G_1-S cyclin" pathway may be altered by number of ways that may liberate E2F continuously leading to uncontrolled cell proliferation. This may happen by:

- In cancer, Rb gene may be inactivated due to mutation or hypermethylation[7].
- Cyclin D1 and CDK4 gene amplification.
- HER-2 gene amplification.

There may be incessant activity of CDK-cyclin complex because of inactivation of CDK inhibitors (CDKI) gene.

Deoxyribonucleic acid replication license and cancer: Deoxyribonucleic acid synthesis is tightly controlled by replication licensing system. At the time of end of mitosis and early G_1 phase, licensing proteins origin recognition complex, Cdc6, Cdt1 and mini-chromosome maintenance (Mcm)2–7 assemble into prereplicative complexes (preRC) and license the "origin" for DNA synthesis in S phase (**Fig. 2.11**). After the entry of the cell into the S phase, the licensing system is shut down so that the cell is unable to reinitiate the DNA replication. Deregulation of the licensing system may be responsible for oncogenesis.[8]

Deoxyribonucleic acid damage, repair and cancer: There are checkpoints in S phase and G_2-M phase to prevent the cell cycle progression in case of DNA damage. Breaking down of double stranded DNA is quickly recognized by ataxia telangiectasia mutated (ATM) and ataxia telangiectasia mutated and Rad 3 related (ATR) proteins (**Fig. 2.12**). In unstressed cell, ATM remains in homodimer state and its kinase activity is blocked. Double-stranded DNA break causes conformational change of ATM and it becomes monomeric active ATM by autophosphorylation.[9] The activated ATM protein gets accumulated at the site of DNA, breaks to act on the substrate. The appropriate localization of ATM monomer and the ATM substrate is modulated and facilitated by group of proteins such as mediator of DNA damage checkpoint 1, 53BP1 (p53 binding protein 1) and BRCA1.[10-13] This active ATM further activates checkpoint *kinase* 2 (CHK2). Similarly, ATR is also activated by break in double-stranded DNA and subsequently phosphorylate critical substrates, such as RAD17 and CHK1.[14]

The activated CHK1 and CHK2 kinases phosphorylate gene regulatory protein p53. In undamaged cell, p53 binds with another protein *mouse double minute 2 (*Mdm2) and the

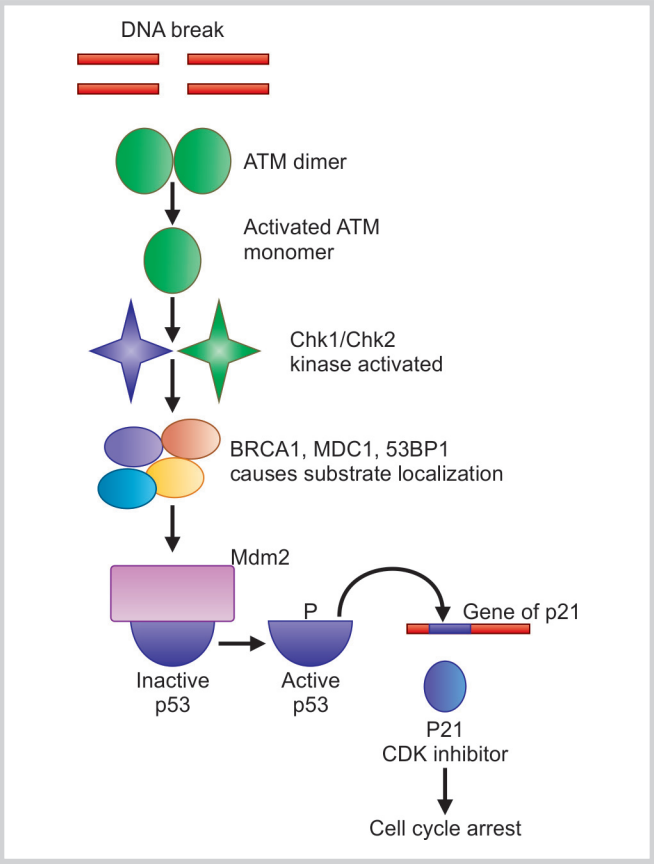

Fig. 2.12: Schematic diagram shows deoxyribonucleic acid damage checkpoint

available concentration of p53 is extremely low. However, phosphorylation of p53 by CHK1 and CHK2 kinase after DNA damage reduces its binding with Mdm2. The marked increase concentration of p53 enhances its ability of stimulation of gene transcription of p21, a CDKI. The p21 protein inactivates G_1-S CDK and S-CDK complexes and blocks cell cycle progression. The arrested cell in cell cycle gets time to repair its damaged DNA. If the DNA is not repaired then the cell undergoes apoptotic death.

The defect in DNA damage checkpoint of the cell cycle may lead to increased mutation of genes and promotion of cancer. A large number of human malignancies are demonstrated due to the mutation of p53 gene.[15] Similarly, increased frequencies of cancer have been noted in BRCA1 mutation and ATM gene defects.[16,17]

CELL PROLIFERATION MARKERS

Assessment of cell proliferation is considered an important biological marker of predicting tumor behavior. There are many cell proliferative markers.[18,19] The counting of mitosis is the oldest and one of the most reliable methods of assessment of cell proliferation activity. There are many other methods to assess the cell proliferation activity, such as DNA FCM to assess the S phase, incorporation techniques, immunocytochemistry of various cell proliferation-related antigens, nucleolar organizer regions counting, etc. These techniques are compared in **Table 2.1**.

Mitotic Index

Mitosis is visible by light microscope in simple routine stain (**Fig. 2.3**), and it can be measured easily.[20,21] The strict morphological criteria should be used for the identification of mitotic figures. The ideal mitotic figures should have: (1) the absence of nuclear membrane, (2) the absence of clear zone in the center, (3) the presence of hairy extension from the side, and (4) basophilic surrounding cytoplasm instead of eosinophilia.

Mitosis count is more predictable and reliable on histology section than cytology smear. Mitosis in tumor can be counted as number of mitotic figures per ten high power fields. However, the field of vision may vary from microscope to microscope. Therefore, it is better to express the mitosis count as number of mitotic figures per 1,000 cells (mitotic index).

Advantage

Easy and more or less reliable.

Disadvantages

- Mitotic activity may vary depending on the fixation of the tissue. If the tissue is not fixed immediately then the cell may complete its mitosis and the mitotic count may be low
- Many other things may simulate mitotic figures such as apoptotic cell, mast cells, degenerated lymphocytes, etc. and mitotic count should be done carefully.

Incorporation Technique

This is based on the incorporation of the labeled nucleotide analogs, such as tritiated thymidine or bromodeoxyuridine (BrDU).[22] The proliferating cells incorporate the labeled nucleotide analogue during DNA synthesis. This can be demonstrated by autoradiography in case of radioactive thymidine and immunohistochemistry in case of BrDU.[23]

Disadvantages

Clinical utility of incorporation techniques is limited. Radioactive thymidine has radioactive hazards. However, presently radioactive thymidine is totally replaced by BrDU, which can be stained by immunohistochemistry.

Deoxyribonucleic Acid Flow Cytometry and Image Cytometry

Deoxyribonucleic acid histogram in DNA FCM helps in the identification of the population of G_0/G_1, S phase and G_2/M phase cells.[24] Similarly image cytometry (ICM) also produces histogram

TABLE 2.1: Different techniques to assess cell proliferation

Technique	*Principle*	*Cell cycle*	*Advantages*	*Disadvantages*
Mitotic count	Estimation of mitotic figures on light microscopy	M phase cells	1. Easy 2. Reproducible 3. Possible on archival material and routine smear or section	1. Mitotic count may vary depending on tissue fixation 2. Mitotic score may vary subjectively 3. Many other things such as apoptotic body and mast cells may simulate mitotic figures
AgNOR	NOR-related-associated proteins are argyrophilic and can be stained with silver. AgNOR is closely related with cell proliferation	All proliferating cells	1. Easy 2. Can be done on archival tissue	1. Counting is to some extent subjective 2. Difficult in daily routine practice
Flow cytometry	DNA stochiometric dye stains DNA of single cells and relative DNA content is measured when the cells are in flow	Measure G_0/G_1, S and G_2/M phase cells separately	1. Rapid 2. Large number of cells can be analyzed. 3. Reproducible	1. Sophisticated technique 2. Target cells cannot be visualized
Image cytometry	DNA stochiometric dye stains DNA and relative DNA content is measured on smear or section	Measure G_0/G_1, S and G_2/M phase cells separately	Target cells can be visualized by light microscopy and manual selection can be done	1. Slow 2. Low resolution of the histogram
Ki67 scoring	Ki67 antigen is expressed in proliferating cells	G_1, S and G_2 phase	1. Easy 2. Reproducible 3. Short half-life of the antigen, so accurate	Strict measuring criteria is needed
DNA precursor base incorporation: BrDU incorporation	BrDu is picked up by proliferating cells in S phase and later can be stained by immunohistochemistry	S phase cell is measured	Gold standard. As it is very accurate and reliable	Not possible in routine life to apply in clinical cases

Abbreviation: BrDU, Bromodeoxyuridine; AgNOR, Argyrophilic nucleolar organizing regions; DNA, deoxyribonucleic acid

that can also help to assess the different population of cells in a cell cycle.[25] In case of DNA FCM and ICM, a stoichiometric dye is used which binds with DNA depending on its amount. The amount of staining is measured which indicates relative DNA content of the cell. Cells in G_0/G_1 phases contain diploid amount (2n) of DNA and cells in G_2/M phases contain tetraploid (4N) amount of DNA. S phase cells contain diploid to tetraploid amount of DNA (2n – 4n).

Advantages

Deoxyribonucleic acid FCM can rapidly analyze large population of cells and therefore gives better resolution of the histogram and reliable result.

Disadvantages

- The specific population of tumor cell is not visible in DNA FCM, and therefore, it gives the result of all cells, including vascular endothelial cells and stromal cells
- This is more sophisticated and costly technique.

Immunohistochemistry

There are various proliferation related antigens that can help in the assessment of cell proliferation.

Proliferating Cell Nuclear Antigen

Proliferating cell nuclear antigen (PCNA) is related with the initiation of DNA replication and cell proliferation.[26] The cells in G_0 and early G_1 phase minimally express PCNA. There is a progressive increase of PCNA in advanced G_1 and S phase cells. This is followed by decreased level of PCNA in G_2 and M phase of cells. There is long half-life of PCNA and therefore, even the cells enter into the G_0 phase,[27] PCNA is demonstrable in the cells. So in some tumors 100% cells may show PCNA positivity. Therefore, PCNA scoring may not always represent the proliferative cell fraction.

Ki67

Ki67 antigen is the most potent antigen to detect proliferating cells. Ki67 antigen is coded by chromosome 10, and it is

expressed in proliferating cells in G1, S and G2 phase of the cell cycle.[28] Originally, Ki67 immunostaining was only possible in frozen sections; however, at present days, other Ki67 monoclonal antibody (MIB-1) works on formalin fixed paraffin section and is reproducible and reliable.[29] Half-life of Ki67 antigen is very short and this antigen is almost totally degraded at the end of mitosis. Therefore, only the actively proliferating cells in cell cycle express this antigen and Ki67/MIB-1 is considered as the best proliferation markers for application in routine use.[30]

Mini-chromosome Maintenance 2–7

Deoxyribonucleic acid replication initiation depends on preRCs that give chromatin license for DNA synthesis during S phase. Mini-chromosome maintenance 2–7 proteins are the components of this complex. The Mcm 2–7 replication licensing factors can clearly distinguish between proliferating and nonproliferating cells, as Mcm 2–7 proteins are present in the nucleus throughout all cell cycle phases.[31]

Nucleolar Organizing Regions

Nucleolar organizing regions (NORs) are the segments of DNA on the short arm of acrocentric chromosomes 13, 14, 15, 21 and 22. They encode genes for ribosomal ribonucleic acid activity, protein synthesis and cell proliferation.[32]

The transcriptionally active NORs have argyrophilic proteins and therefore can be demonstrated by silver staining technique (**Fig. 2.13**). So the term argyrophilic nucleolar organizing regions (AgNORs) has been developed. The AgNORs organizing regions assessment is intimately related with rate of cell proliferation.

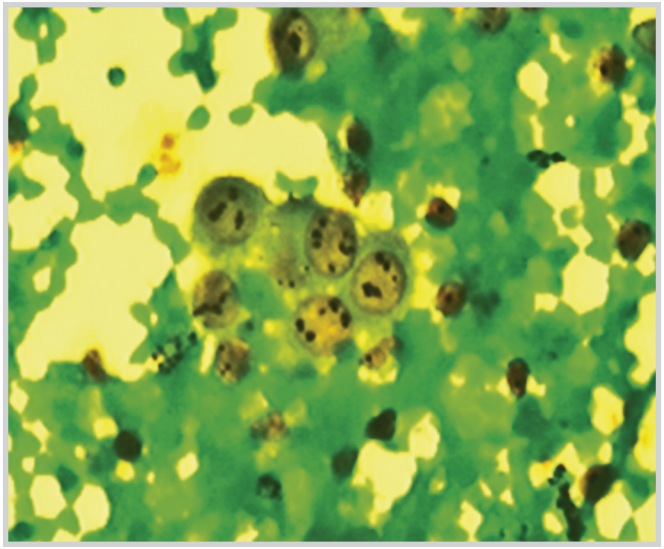

Fig. 2.13: Nucleolar organizer regions are demonstrated by silver staining technique (Argyrophilic nucleolar organizing regions stain X OI)

Advantages

Easy technique to measure cell proliferation.

Disadvantages

- Noncycling cell may also synthesize protein and may show AgNORs. Therefore, baseline AgNOR score measurement is needed
- The AgNORs scoring may vary depending on thickness of the section
- Due to aggregation and segregation of NORs during the mitotic cycle AgNOR scoring may vary artifactually even though the total number of NORs in the nucleus is constant.

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CHAPTER 3

Cellular Reaction to Injury and Cell Death

Chapter Contents

INTRODUCTION

Various external and internal stimuli can cause cell injury. These injurious stimuli may be gross external or from intricate molecular level. The different causes of cell injury have been highlighted in **Box 3.1**.

Hypoxia or deprivation of oxygen is the common cause of cell injury. Hypoxia may be due to loss of vascular supply or may be due less oxygen carrying capacity of blood.

BOX 3.1 **Different causes of cell injury**

- Hypoxia
- Physical agents: Heat, cold, electrical injury, radiation effect, pressure effect
- Chemical agents and drugs: Different chemical poisons, insecticides, drugs, etc.
- Nutritional causes: Nutritional imbalances such as protein calorie malnutrition, hyperlipidemia, vitamin deficiency, etc.
- Infectious agents: Virus, bacteria, parasites, etc.
- Immunological diseases: Autoimmune diseases, inborn immune disorders
- Genetic defects: Inborn errors of metabolism, chromosomal abnormalities, etc.

In response to various stresses, individual cell responses variedly. The exact cellular response to stress depends on: type, intensity, and duration of injury or stress. The cell initially tries to maintain a steady state called homeostasis. If the stress or injury to the cell is more, the cell takes an adaptive state, such as the number of the cells may be increased or decreased or the cell may change its type. If the cells fail to adapt to the increased stress or if there is an acute internal or external stimuli, the cells undergo injury. Cellular injury may be reversible or irreversible. In case of irreversible injury, the cell dies, which is the ultimate fate of any cell.

CELLULAR ADAPTATION

The various types of cellular adaptations are (**Fig. 3.1**):

- Hyperplasia
- Hypertrophy
- Atrophy
- Metaplasia.

Hyperplasia

The term hyperplasia indicates increase in number of cells in a tissue or organ. This causes an overall increase of volume or weight of the tissue due to cell proliferation. Hyperplasia occurs

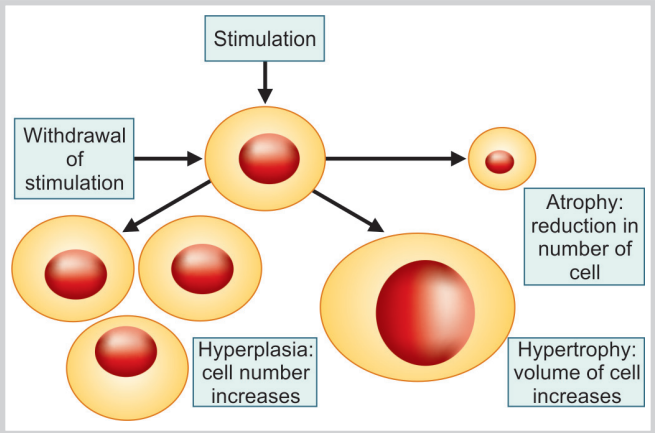

Fig. 3.1: Schematic diagram showing different types of adaptation

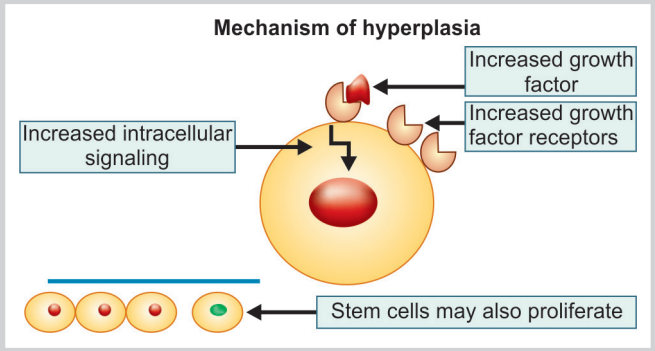

Fig. 3.2: Schematic diagram showing mechanisms of hyperplasia. There may be increased: (1) growth factor; (2) growth factor receptors; (3) intracellular signaling. Stem cells may also show proliferation

when the cells are capable of synthesizing their DNA and are able to replicate. Hyperplasia is usually caused due to (1) increased growth factors, (2) increased growth factor receptors, or (3) increased intracellular signaling (**Fig. 3.2**). At times, the stem cells may also proliferate to take part in hyperplasia. Hyperplasia often gives rise to neoplastic proliferation, particularly in case of atypical hyperplasia.

In light microscopy on cytology smear, it is difficult to comment on hyperplasia. However, if the cellularity of the smear is more than normal (such as noted in follicular hyperplasia of the thyroid), the possibility of hyperplasia may be suggested (**Fig. 3.3**).

Hypertrophy

The term hypertrophy indicates increase in volume of the cell leading to increase in organ size. No cellular proliferation occurs in case of hypertrophy. The individual cells may be larger in size with enlarged nuclei. Hypertrophy is difficult to assess by light microscopy on cytology smears.

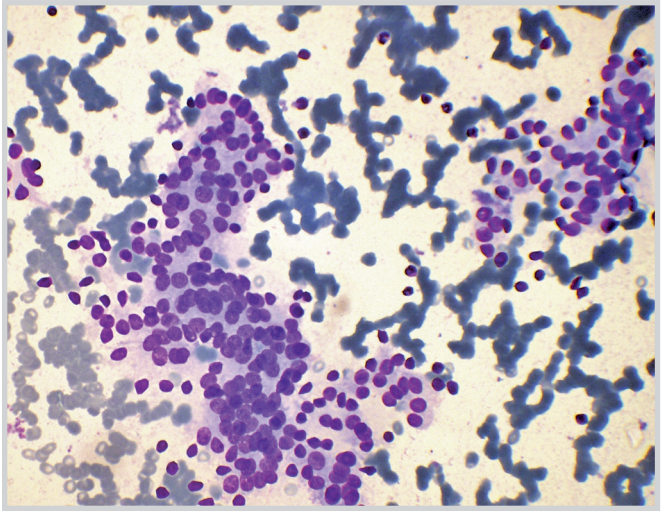

Fig. 3.3: Abundant thyroid follicular cells in case of thyroid follicular hyperplasia (May Grunwald Giemsa stain X MP)

Atrophy

Atrophy means reduction in the number of cells leading to shrinkage of tissue or organ. The causes of atrophy may be variable such as inadequate nutrition, loss of endocrine stimulation, loss of innervations, decreased workload, pressure effect or aging. By light microscopy, epithelial atrophy can be recognized on a cytology smear, such as atrophic cellular pattern on cervical cytology.

Metaplasia

Metaplasia means replacement of one type of mature cell type to another type of mature cell type. This replaced epithelium may better resist the adverse circumstances. The most common type of epithelial metaplasia is columnar to squamous metaplasia. This may be noted in the squamocolumnar junction of the cervix or in endocervical glands (**Fig. 3.4**), in the bronchial respiratory lining epithelium, and in the salivary excretory duct. Metaplasia from squamous to columnar epithelium may occur in Barrett's esophagus, which is a known precancerous condition.

In case of metaplasia, the stem cell is reprogrammed to develop a different type of tissue rather than the change of phenotypic expression of the mature epithelium. Possibly one tissue-specific gene becomes off, and the another tissue-specific gene becomes on.

REVERSIBLE CELL INJURY

When a cell faces stress or acute stimulation, which is beyond the limit of adaption, the cell suffers injury. If the acute assault is withdrawn in a short period of time then the cell restores its structure and function. However, it is still unknown at which point reversible injury will go to "the point of no return" or irreversible pathway.

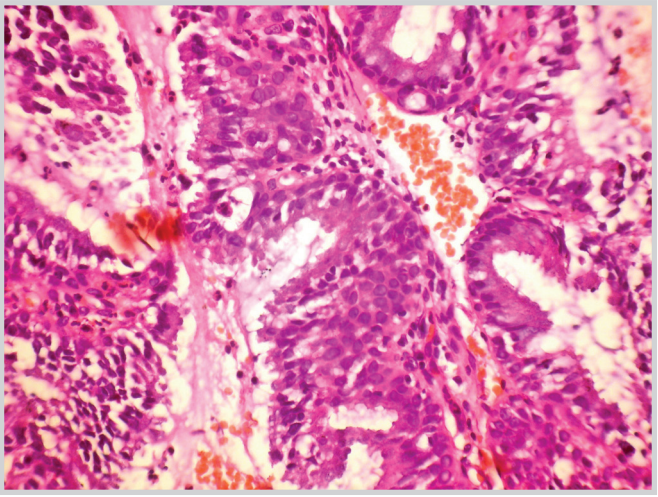

Fig. 3.4: Squamous metaplasia of endocervical glands (hematoxylin and eosin X MP)

Morphological Changes of Reversible Injury

Hydropic Swelling

In case of hydropic swelling, the cells are swollen with large pale cytoplasm and centrally placed nucleus. Cytoplasm often shows multiple small vacuoles. By electron microscopy, the following changes are seen:

- Blunting and distortion of microvilli of plasma membrane
- Dilatation of endoplasmic reticulum
- Swelling of mitochondria
- Disaggregation of fibrillar and granular components of the nucleolus.

IRREVERSIBLE INJURY

The ultimate fate of all cells is death. Cell death can be divided in two basic forms: (1) Programmed cell death (PCD) and (2) accidental cell death by necrosis. The PCD is now classified as type I (apoptosis), type II (autophagy), and type III (programmed necrosis).[1,2]

For a long time, the term "apoptosis" was used as a general term to describe PCD, and the concept of nonapoptotic PCD was deliberately ignored. Because of the advancement in molecular technology, other forms of nonapoptotic PCD are getting increased attention.

Type I PCD or apoptosis is critically important for the developmental morphogenesis, tissue homeostasis, and defense against pathogens. This phenomenon is highly conserved and well-described at both genetic and biochemical levels.

Type II autophagic cell death[3] is a major cellular catabolic pathway for degradation of long-lived proteins and cytoplasmic organelles. Thus autophagic PCD helps in maintenance of proteins and cytoplasmic organelles and enhances cell survival. It occurs in tissue and cell remodeling, starvation, and cell death. Type III programmed necrosis is a distinct entity. It is a passive process that affects large population of cells or group of cells by combined use of selected effectors along with other cell death outcomes. It is called as programmed necrotic death because the cellular signaling pathways lead to necrosis in response to specific effectors rather than by simple accident.

APOPTOSIS OR TYPE I PROGRAMMED CELL DEATH

The term apoptosis is derived from the Greek word *Apo* (means from) and *ptosis* (means fall). This suggests "leaves falling from a tree". Apoptosis is one of the major ways to suicidal death and it is distinctly different than necrosis (**Table 3.1**). The predominant biochemical events in apoptosis are activation of intracellular proteases and step ladder type of fragmentation of internucleosomal DNA. The various cell surface molecules are altered in the apoptotic cells that help in the immediate recognition of the apoptotic cells by neighboring cells that can phagocytose them. This orderly elimination of the apoptotic cells prevents any inflammatory reaction.

Morphology of Apoptosis

Apoptosis typically occurs in individual cells without any surrounding inflammatory reactions. The following morphological changes are noted in apoptotic cells[4,5] (**Box 3.2**).

Nucleus

- The chromatin becomes pyknotic. It condenses and aggregates in the inner surface of the nuclear envelope. This often gives half-moon or sickle-like appearance of the chromatin

TABLE 3.1: Distinguishing points between apoptosis and necrosis

Features	*Apoptosis*	*Necrosis*
Energy	Active process and energy is required	Passive process and no ATP is required
Nucleus	Condensed peripheral chromatin	Fragmented chromatin
Cell size	Cell shrinkage	Cell swollen
Death pattern	Single cell death	Group of cells dye
Inflammatory response	No inflammation	Inflammation
DNA fragmentation	Regular internucleosomal 180 bp length	Irregular fragments
Cell membrane	Preserved membrane	Fragmented membrane

Abbreviations: ATP, Adenosine triphosphate; DNA, Deoxyribonucleic acid

BOX 3.2 Morphological changes of apoptosis

Nuclear changes:
- Chromatin condensation and aggregation
- Nuclear convolution and multiple budding
- Nuclear fragments are covered by double layer of membrane

Cytoplasmic changes:
- Cytoplasm condenses
- Extensive protrusion of the cell surface
- The protuberance of the cell surface is separated and sealed by the plasma membrane

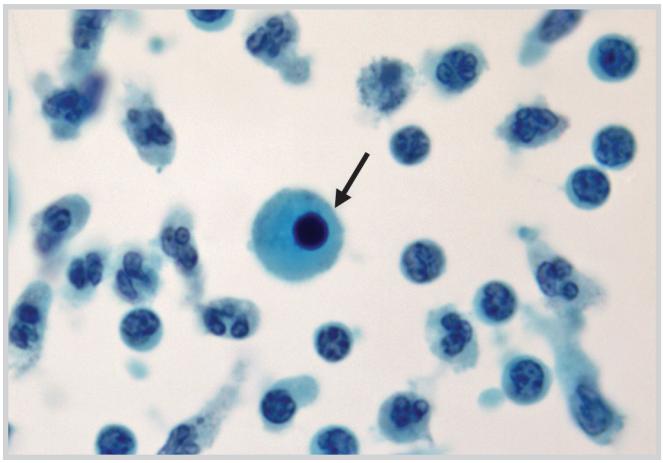

Fig. 3.5: Cell with moderate amount of cytoplasm and dense nucleus in case of an apoptotic cell (arrow) (Papanicolaou's stain X HP)

- In most of the cases nuclear outline becomes convoluted
- Severe nuclear convolution causes multiple budding and nuclear fragments of different sizes (budding phenomenon)
- Each nuclear fragment is covered by a double layer of membrane (**Figs 3.5** and **3.6**).

Cytoplasm

- Cytoplasm condenses and extensive protrusion or blebbing appears on the cell surface
- Numerous clear vacuoles appear in the cytoplasm
- The protuberance on the cell surface is separated and sealed by the plasma membrane forming multiple membrane-bound apoptotic bodies
- There is no swelling of mitochondria.

These apoptotic bodies are readily phagocytosed and destroyed by the neighboring cells or macrophages. During the process of degradation, the apoptotic bodies can be visualized if the cell contains remarkable condensed chromatin. Overall tissue architecture is not disturbed in the apoptotic process, as there is death of single isolated cells.

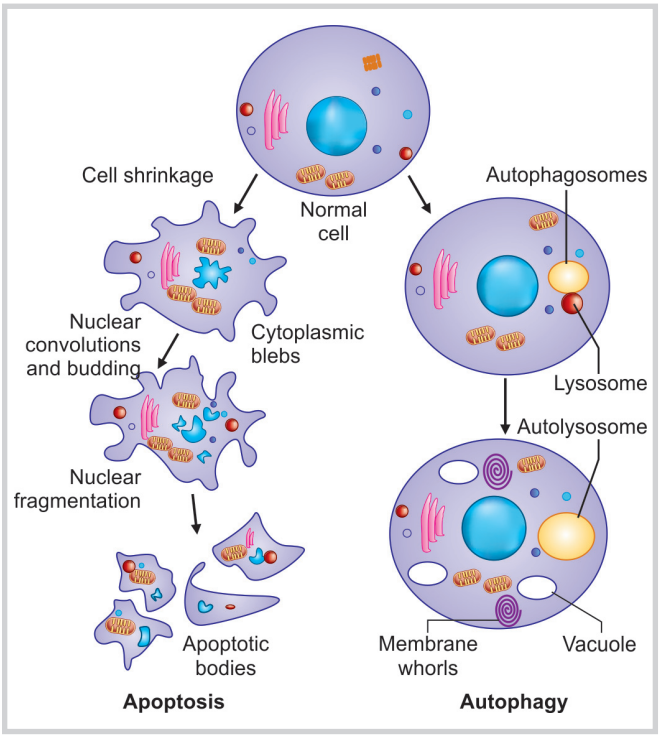

Fig. 3.6: Schematic diagram showing morphological changes in apoptosis and autophagy

Molecular Pathways of Apoptosis

There are two major molecular pathways of apoptosis: (1) death receptor pathway and (2) mitochondrial pathway[6,7] (**Box 3.3**).

Death Receptor Pathway

Death receptors are the members of tumor necrosis factor-receptors (TNF-receptors) gene super family and are located on the cell surface. There are two parts of death receptor, an intracytoplasmic domain known as "death domain" and cysteine-rich extracellular domain. The "death domain" plays an important role to trigger intracellular apoptotic machinery. The most well-known death receptors are CD95 (also called Fas or Apo1) and type 1 TNF receptor 1 (TNFR1). The ligands that activate these receptors are structurally related molecules. CD95 ligand (CD95L) binds to CD95; TNF and lymphotoxin alpha bind to TNFR1 (**Fig. 3.7**).

Binding of death receptor with the ligand recruits the adaptor protein Fas-associated death domain (FADD). This FADD protein further activates caspase 8 and this subsequently activates the caspase 3, the main executioner enzyme.[8] This activated caspase 3 cleaves the various substrates.

Mitochondrial Pathway

B-cell lymphoma 2 (BCL-2) family members play significant role in the mitochondrial pathway of apoptosis.[6,9] Increased intracellular

BOX 3.3 Molecular pathway of apoptosis

Death receptor pathway:
- Death receptor:
  - Intracytoplasmic domain ("death domain")
  - Cysteine-rich extracellular domain
  - CD 95 (fas or apo 1)
  - Type 1 TNF receptor (TNFR1)
- CD95 ligand binds to CD95; TNF binds to tnfr1
- Binding of death receptor with the ligand recruits the adaptor protein FADD
- FADD protein further recruits caspase 8 and this subsequently activates the caspase 3

Mitochondrial pathway:
- Increased intracellular reactive oxygen species, DNA damage or other intracellular factors inhibit antiapoptotic proteins BCL-2/BCL-x
- Increased proapoptotic proteins Bak, Bax and Bim
- Increased mitochondrial membrane permeability releases cytochrome C
- Activation of caspase 9

Final phase
- Caspase 8 and caspase 9 subsequently activates the caspase 3 which act as executioner enzyme

Abbreviations: TNFR, tumor necrosis factor receptor; FADD, fas-associated death domain; BCL-2, B-cell lymphoma 2

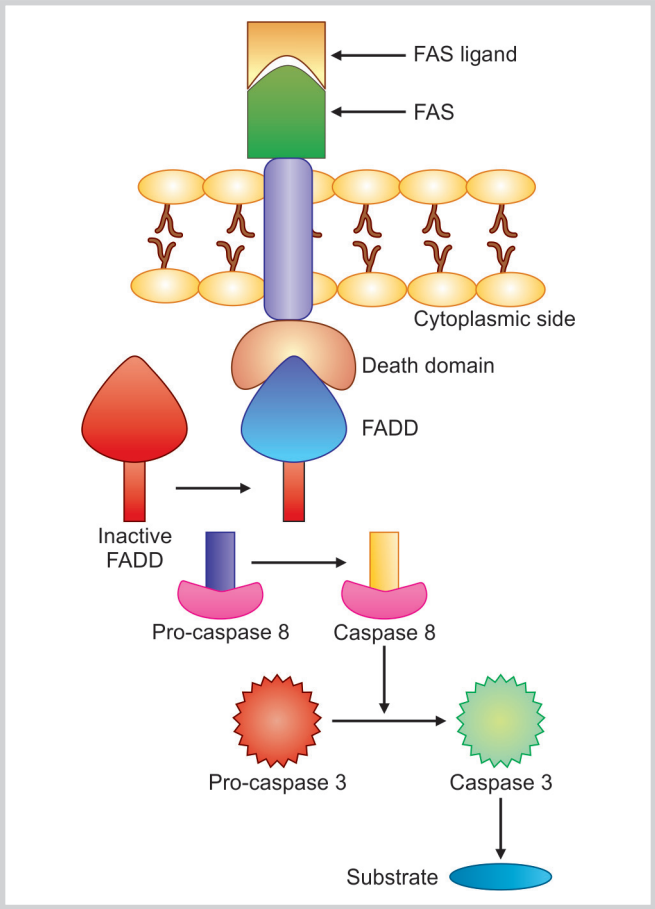

Fig. 3.7: Schematic diagram showing mechanism of death receptor pathway of apoptosis

reactive oxygen species, DNA damage or other intracellular factors initiate this pathway by inhibiting antiapoptotic proteins BCL-2/BCL-x. The decreased level of BCL-2/BCL-x is replaced by proapoptotic proteins Bak, Bax, and Bim. The multiple stimuli such as proapoptotic proteins (Bak, Bax, and Bim), Ca^{++} overload, oxidants and active caspases increase mitochondrial membrane permeability and release caspase activation protein cytochrome C and apoptosis inducing factor (AIF) from the mitochondria to the cytoplasm. Cytochrome C binds with apoptosis activating factor that further helps in activation of caspase 9 and initiates the proteolytic cascade leading to apoptosis (**Fig. 3.8**).[9]

Final Phase and Biochemical Changes

Activated caspase 8 generated from the death receptor pathway and activated caspase 9 generated from the mitochondrial pathway both ultimately mobilize caspase 3, 6 and 7 proteases.[7] These executioner caspases act on numerous cellular components (**Box 3.4**).

Protein Breakdown

Activated caspases (particularly caspase 3) cleave various proteins such as cytoskeletal proteins, nuclear matrix proteins and proteins involved in DNA transcription, replication and repair.

Deoxyribonucleic Acid Breakdown

Caspase 3 activates the cytoplasmic deoxyribonuclease which characteristically breaks internucleosomal DNA into segments

BOX 3.4 Biochemical pathway of apoptosis

Proteins breakdown:
- Activated caspases (particularly caspase 3) cleave:
  - Cytoskeletal proteins
  - Nuclear matrix proteins
  - Proteins involved in DNA transcription, replication and repair

DNA breaksdown:

Caspase 3 activates the cytoplasmic deoxyribonuclease and breaks internucleosomal DNA into segments into multiples of 185 bp

Phagocytosis:
- The caspase produces the loss of phospholipid asymmetry and the exposure of PtdSer on the outer surface of the plasma membrane
- PtdSer acts as a ligand for the SR of macrophages
- The interaction between SR and PtdSer helps in phagocytosis

Abbreviations: PtdSer, phosphatidylserine; SR, scavenger receptors

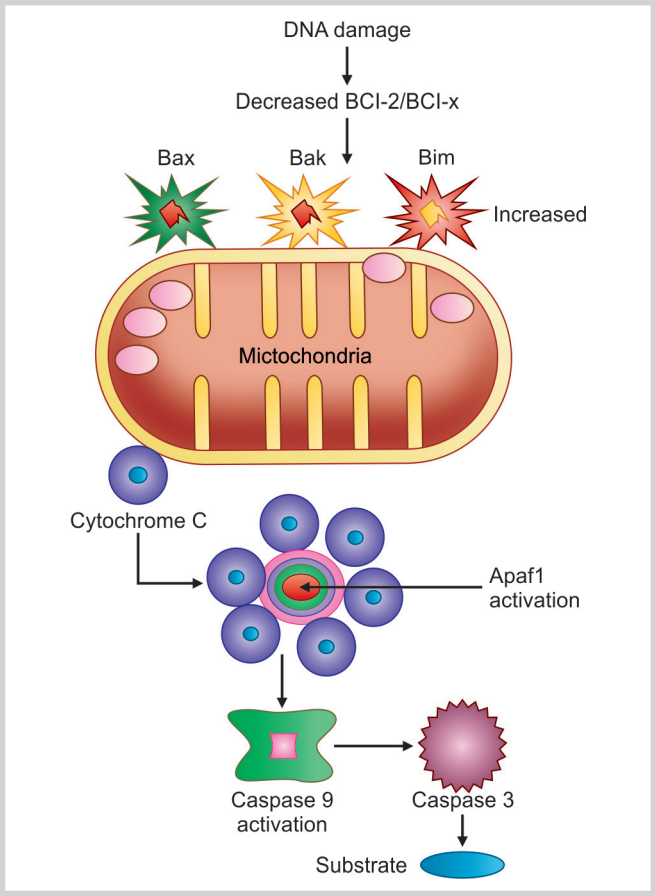

Fig. 3.8: Schematic diagram showing mitochondrial pathway of apoptosis

that are multiples of approximately 185 bp. This step ladder type of fragments of DNA can be demonstrated by gel electrophoresis.

Removal of the Apoptotic Bodies

The apoptotic bodies are readily phagocytosed by the neighboring cells or macrophages to prevent the release of potentially noxious or immunogenic materials from the apoptotic cells and initiation of inflammation. This preserves the integrity and function of the surrounding tissue. The apoptotic cells express certain unique signals to the phagocytes so that the phagocytes recognize the apoptotic bodies.[10] The caspase activity in the apoptotic process causes certain changes in the plasma membrane, such as the loss of phospholipid asymmetry and the exposure of phosphatidylserine (PtdSer) on the outer surface of the plasma membrane.[11] Phosphatidylserine acts as a ligand for the scavenger receptors (SR) of macrophages and the interaction between SR and PtdSer helps in phagocytosis of the apoptotic body. The phagocytosed apoptotic bodies are digested further by lysosomal enzymes of the phagocytes.

Apoptosis in Physiological Conditions

The apoptosis occurs in various physiological conditions.

Embryogenesis

Apoptosis takes part key role in embryogenesis by the programmed death of specific cell type during the time and spatial level.

Homeostasis of the Cell Population

Apoptosis maintains the total amount of viable functioning cells by deletion of the cells.

Hormone-dependent Involution

Apoptosis plays key role in the hormone-dependent regression in various organs, such as regression of ovarian follicles, prostate shrinkage after testicular removal, etc.

Cell Death in Infection

Apoptosis occurs as a defense mechanism by cytotoxic-T cells during viral infection.

Prevention of Autoimmunity

With the help of apoptosis, the autoreactive lymphocytes are eliminated from the body.

Apoptosis in Diseases

There are widespread clinical implications of apoptosis as too little or too much apoptosis, both causes diseases (**Box 3.5**).

BOX 3.5 **Apoptosis in diseases**

- Diseases associated with less apoptosis and increased cell survival:
  - Apoptosis and cancer: Follicular lymphoma, breast cancer, prostate cancer, etc.
  - Immunological disorders: Autoimmunity, such as systemic lupus erythematosus, immune-mediated glomerulonephritis
  - Apoptosis in viral infection: Virus-infected cells do not undergo apoptosis.
- Diseases associated with more apoptosis and decreased cell survival:
  - Virus-induced lymphocyte depletion: In HIV infection
  - Apoptosis in neurodegenerative disorders: Parkinson's disease, Alzheimer's disease, spinal muscular atrophy, retinitis pigmentosa, and amyotrophic lateral sclerosis
  - Apoptosis in neurodegenerative disorders: Aplastic anemia, myelodysplastic syndromes, anemia associated with chronic disease, and chronic neutropenia.

Apoptosis and Cancer

Death by apoptosis plays a significant defense mechanism against malignant transformation.[12,13] The failure of apoptosis causes uncontrolled proliferation of the cells and therefore produces the ideal environment of genetic instability and mutation. Majority of cancers, such as follicular lymphoma, breast cancer, and prostate cancer have defects in apoptotic machinery. The predominant defects are the increased expression of antiapoptotic protein BCL-2 and mutation of tumor suppressor gene p53. The mutation of p53 gene causes defective p53 protein production and failure of apoptosis during DNA damage.[14-17]

Immunological Disorders

Apoptosis is essential for the removal of potentially autoreactive T and B lymphocytes during the development and for the removal of excess cells after the completion of an immune response. Failure of deletion of such cells may increase the susceptibility of autoimmunity such as systemic lupus erythematosus, immune-mediated glomerulonephritis, etc.[17,18]

Apoptosis in Viral Infection (Herpes, Adenovirus and Poxvirus Infection)

Virus-infected cells undergo apoptosis and thus prevent the spread of the infection. To combat the host's defensive mechanism, many viruses have developed ways to disrupt the apoptotic machinery within the infected cell.[17]

Diseases Associated with More Apoptosis and Decreased Cell Survival

The various genetic and acquired conditions may cause excessive cell death by enhancing the signal for induction of apoptosis or by decreasing the threshold of induction of apoptosis.

Virus-induced Lymphocyte Depletion

In AIDS, the HIV infection causes selective depletion of CD4+ T lymphocytes by inducing apoptosis and destruction of the host's body defense.[19]

Apoptosis in Neurodegenerative Disorders

The gradual loss of a specific set of neurons by apoptosis occurs in large numbers of neurodegenerative disorders such as Parkinson's disease, Alzheimer's disease, spinal muscular atrophy, retinitis pigmentosa, and amyotrophic lateral sclerosis.[17]

Apoptosis in Hematological Disorders

Increased apoptosis and cell destruction are related to a wide number of hematological disorders such as aplastic anemia, myelodysplastic syndromes, anemia associated with chronic disease, and chronic neutropenia.[20]

Detection of Apoptosis

There are various ways to detect apoptosis.

Morphology

The apoptotic cells can be identified in light microscopy on routine hematoxylin and eosin stain smears or on histology sections. The apoptotic cells have small condensed chromatin (**Fig. 3.5**). The apoptotic cells can also be demonstrated by fluorescent microscope after doing acridine orange stain. The DNA in the apoptotic cell looks brightly green by acridine orange stain, whereas DNA in normal cell looks yellow green.[21]

Gel Electrophoresis

On gel electrophoresis, step ladder-like pattern is seen in apoptosis due to internucleosomal DNA fragmentation. This pattern is not very specific for apoptosis and the morphology of the cell cannot be demonstrated by electrophoresis.

Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick End Labeling

In this technique, the 3' OH ends of the small DNA fragments are coupled with labeled biotinylated deoxynucleotides by using DNA polymerase I or terminal deoxynucleotidyl transferase as catalyst. Only the apoptotic cells incorporate the biotinylated deoxynucleotides into their DNA fragments and then are demonstrated by simple immunohistochemistry on light microscopy.[22] This technique can be done on histology section or cytology smear and the exact quantification of the apoptotic cells are possible in clinical samples.

Flow Cytometry

Deoxyribonucleic acid fluorochrome dye is used to stain DNA stoichiometrically. The relative DNA content of the cells is measured. The apoptotic cells contain less amount of DNA and are readily identified by flow cytometry. Annexin V binds with PtdSer on the cell membrane of apoptotic cell and helps in detection of apoptotic cells with the help of flow cytometry.[23]

AUTOPHAGY

The word "autophagy" is derived from the Greek word "*Auto*" means self and "*phagy*" means "to eat" (**Box 3.6**). Autophagy is defined as the PCD by which cells recycle their own nonessential, redundant, or damaged organelles.[24] It is the active physiological process by which the cells actually recover the essential molecules, such as amino acids and fatty acids. These are further used for protein synthesis or ATP production. Thus, autophagy

BOX 3.6 Autophagy

Definition: The programmed cell death by which cells recycle their own nonessential, redundant or damaged organelles
Types of autophagy: (1) microautophagy (2) macroautophagy and (3) chaperone-mediated autophagy

Morphological changes in autophagic death:
- Autophagosomes formation: Double membrane vesicles containing engulfed cytoplasmic organelles
- Autolysosome: Fusion of autophagosome and lysosome
- Loss of organelles and cytoplasmic vacuoles
- Nuclear chromatin condensation and destruction of nucleus

Molecular pathway:
- Controlled by a group of autophagy-related genes
- Class III Pi3-kinase is the major lipid signal that generates Pi3-P
- Pi3-P helps in the autophagic vesicle formation

clinical implications of autophagy:
Autophagy probably acts as suppressor of neoplasm

TABLE 3.2: Comparison of apoptosis and autophagy

	Apoptosis	*Autophagy*
Programmed death	Yes	Yes
Role of caspase	Yes	No
Cytoskeletal breakdown	Early part	Later or terminal part
Organelles	Not destroyed in early part	Destroyed in early part
Light microscopy	Visible	Not visible, only demonstrable in electron microscopy
Role	Direct role in neoplasia. Too less apoptosis causes cancer	Autophagy plays as tumor suppressor role

maintains an adequate nutrient level at the time of stress or starvation. Cell death by autophagy is caspase-independent programmed death. **Table 3.2** shows the differentiating points between autophagy and apoptosis.

Types of Autophagy

Based on mechanism of lysosomal interaction with cargo, autophagy has been classified into three types: (1) microautophagy, (2) macroautophagy, and (3) chaperone-mediated autophagy.[25]

Microautophagy

In case of microautophagy, the lysosomal membrane invaginates and then engulfs the cargo.

Macroautophagy

Here the autophagosome, the double membrane structure, envelops the cargo and then fuses with the lysosome to form phagolysosome.

Chaperone-mediated Autophagy

Here, the heat shock protein delivers its substrate to the lysosome.[26]

Morphological Changes in Autophagic Death

The autophagic cell death is best visualized by electron microscopy. There are double membrane vesicles containing engulfed part of the cytoplasm and also cytoplasmic organelles such as mitochondria and endoplasmic reticulum. This vesicle is known as autophagosomes. Later on, these vesicles or autophagosomes fuse with the lysosomes to form autolysosome and the contents are degraded (**Fig. 3.6**). The nucleus of the cell also shows condensation and chromatin appears as clumped on the nuclear membrane. However, no DNA fragmentation or formation of the apoptotic bodies is seen.

Molecular Basis of Autophagy

The autophagy is controlled by a group of autophagy-related genes (ATG genes) almost similar to that of yeast. These genes produce at least 27 proteins that are involved in the execution of autophagy such as vesicle enucleation, autophagic vesicle fusion to late lysosome and cargo degradation.

Class III phosphatidylinositol 3 (PI3)-kinase is the major lipid signal that generates phosphatidylinositol 3-phosphate (PI3-P). This PI3-P plays a key role in the autophagic vesicle formation. A protein complex consisting of ATG 6 (Vps30 or Beclin-1 in mammalians) regulates the activity of the Class III PI3-kinase (**Fig. 3.9**).[27] Expansion of autophagic vesicles is formed by two ubiquitin-like pathways:

1. Autophagy-related genes 12 pathway involving ATG 5, 7 and 10
2. Autophagy-related genes 8 pathway involving ATG 7, 3 and 4.

Clinical Implications of Autophagy

The exact role of autophagy in cell death is a debatable issue. Large numbers of autophagic vesicles are noted in dying cells. However, it is still not clearly known that this observation is the cause or effect. The destruction of the large number of cellular organelles may have the detrimental effect of the cells or one can assume that the increased number of autophagosomes facilitates the cell

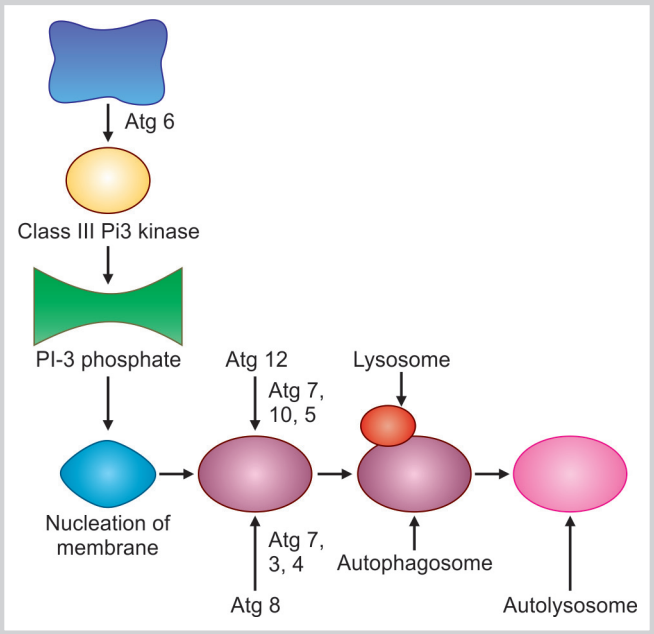

Fig. 3.9: Schematic diagram showing molecular mechanism of autophagic cell death

death. It has been shown that the deletion of key autophagy genes accelerates cell death.[28] This indicates that autophagy helps in cell survival rather than death. Autophagy probably acts as a suppressor of neoplasm. Many oncogenes suppress autophagy and the tumor suppressor genes promote autophagy.[29] Mutation of autophagy genes is noted in hepatocellular carcinomas, gastric and colorectal carcinomas.[30,31]

NECROSIS

The word necrosis is derived from the Greek word "*nekros*" for corpse. It usually occurs due to ischemia or hypoxia, such as in myocardial infarction. In neoplasm, necrosis occurs when the cells proliferate rapidly without adequate angiogenesis. Necrosis is originally considered as accidental cell death. However, accumulating evidences suggest that necrosis is more systematic and ordered.[7]

In case of programmed necrosis, the cell death occurs in sequential events due to the effect of multiple signaling pathways rather than a single well-described signaling cascade.

Compared to caspase-induced death in apoptosis and lysosomal enzyme-induced death in autophagy, the death in programmed necrosis is due to enhanced production of reactive oxygen species, ATP depletion, proteolysis by calpains and cathepsins and dysfunction of mitochondria.

Morphological Changes in Necrosis

Cytoplasmic Changes

Cytoplasm of the necrotic cells shows extensive vacuolization and intense eosinophilia on hematoxylin and eosin stained smears.

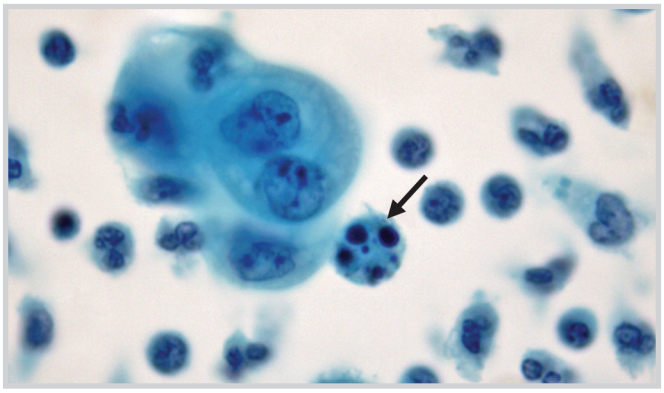

Fig. 3.10: Cytology smear showing typical karyorrhexis of the nucleus (arrow) (Papanicolaou's stain X HP)

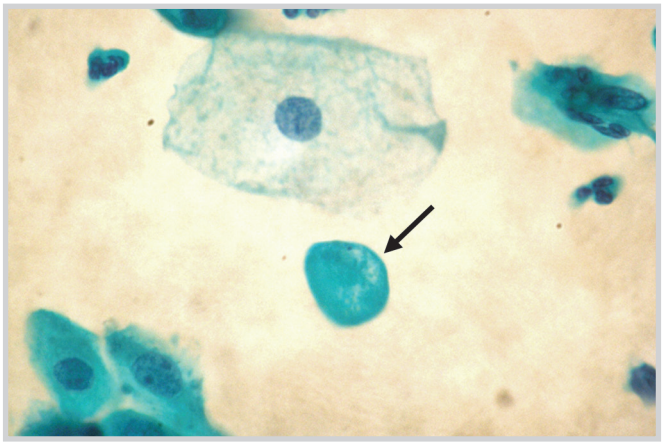

Fig. 3.11: Cytology smear showing typical karyolysis of the nucleus. The nucleus of the cell is almost dissolved (arrow) (Papanicolaou's stain X HP)

Nucleus

Nucleus of the necrotic cell shows following changes (**Fig. 3.10**):

- Pyknosis: Nucleus becomes small and deeply basophilic with clumped chromatin
- Karyorrhexis: Nucleus undergoes fragmentation and smaller fragments scattered within the cytoplasm (**Fig. 3.10**)
- Karyolysis: There is progressive loss of chromatin staining and basophilia of the nucleus fades out (**Fig. 3.11**).

The ultrastructurally necrotic cell is characterized by early swelling of intracellular organelles, such as mitochondria, endoplasmic reticulum, and Golgi apparatus, aggregated cytoskeletal elements and loss of plasma membrane integrity.

Necrosis is Programmed

During cell destruction by necrosis, high mobility group box 1 (HMGB1) protein is released from the nucleus. This HMGB1 protein enters in the circulation and stimulates the innate immune cells as an alarm. Moreover, the necrosis may occur if certain cell surface receptors (TNFR or Fas) are stimulated as an example, high concentration of TNF may cause necrosis of the hepatocytes.[32]

A large number of evidences suggest that the necrosis is not an accident and is regulated by different proteins, such as TNF-related apoptosis-inducing ligand, TNFR1 associated death domain, TNF receptor-associated factor 2, Jun N-terminal kinase 1, receptor interacting protein 1, X-ray repair cross-complementing protein 1, AIF, calpains, Bax, or Dynamin-related protein 1.[33-35]

INFLAMMATION

Inflammation is the response of tissue reaction to exogenous or endogenous stimuli in vascularized tissue. It is fundamentally a protective response of the body to get rid of the pathogenic insult and to remove the injured dead tissue so that healing can occur. Specific cells accumulate in the site of insult to fight with the infective agents. Vasodilatation and increased vascular permeability produce excess fluid in the site of inflammation and dilute the causative agents. Neutrophils and macrophages remove the dead cells and help in healing.

Inflammation is classified as:
- Acute inflammation
- Chronic inflammation
- Granulomatous inflammation.

Acute Inflammation

It is characterized by direct injury to tissue leading to necrosis, damage of larger vessels, and infiltration by polymorphonuclear leukocytes. In acute inflammation, a series of changes occur: (1) Transient and rapid constriction and dilatation of blood vessels, (2) increased vascular permeability leading to local edema in the tissue, (3) release of soluble mediators (histamine, serotonin, prostaglandin, nitric oxide) in the site of inflammation, and (4) infiltration of neutrophils. In most of the time, acute inflammation heals; however, the other outcomes of acute inflammation are scar, abscess formation, and transition to chronic inflammation.

Chronic Inflammation

Chronic inflammation is always of prolonged duration and is associated with acute inflammation and tissue damage. In case of chronic inflammation, the predominant inflammatory cells are mononuclear cells such as lymphocytes, plasma cells and macrophages. Tissue destruction continues with persistent action of the causative agents. There is usually disordered attempt to restore the tissue architecture by angiogenesis and fibrosis.

Granulomatous Inflammation

Granulomatous inflammation is a special type of chronic inflammation characterized by focal collection of activated macrophages resembling epithelial cells (known as epithelioid cells). In fact, granulomatous inflammation is the protective response to chronic inflammation by which the persistent causative agent is removed to protect the host.

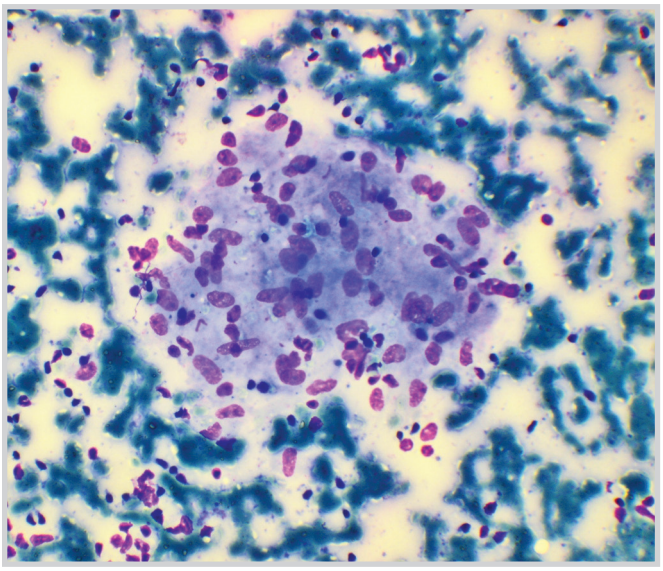

Fig. 3.12: Epithelioid cell granuloma in a case of granulomatous inflammation (May Grunwald Giemsa stain X MP).

Morphology

The hallmark of granulomatous inflammation is epithelioid cell granuloma (**Fig. 3.12**). This is a well-circumscribed nodular lesion formed by epithelioid cells, lymphocytes, and plasma cells. Epithelioid cells have abundant cytoplasm with indistinct cytoplasmic margin. The nucleus is oval to elongated with a blunt end having fine chromatin and inconspicuous nucleoli. Epithelioid cells may often fuse together to form multinucleated giant cells. These giant cells are large 40–50 microns in diameter with 15–20 nuclei. The nuclei may be arranged haphazardly in the cell as we see in foreign body granuloma or they may be arranged around the periphery of the cell in a horse shoe-shaped manner giving rise to the typical appearance of Langhans type of giant cell.

Granuloma may be further classified as:
Granuloma with necrosis: Here necrosis is often seen in association with the epithelioid cell granulomas. This is a typically seen mycobacterium infection.

Granuloma without necrosis: Here no necrosis is seen in association with granulomas. This type of reaction is typically seen in sarcoidosis.

Causes of Granulomas

Box 3.7 highlights the various causes of granulomatous reaction in the body.[36,37]

Morphologically, it is difficult to identify the exact causes of granulomatous reaction. However, the presence of foreign bodies within the giant cells may indicate foreign body granulomas. At times, special stain may help identify the bacteria or fungi.

BOX 3.7 **Causes of granulomatous reaction**

- Infective agents:
  - Bacterial:
    - *Mycobacterium tuberculosis*
    - *Mycobacterium Leprae*
  - Spirochetes
    - *Treponema pallidum*
  - Fungus: *Histoplasma capsulatum, Aspergillus, Cryptococcus,* etc.
- Autoimmune diseases:
  - Rheumatoid arthritis, giant cell arteritis, etc.
- Foreign body granulomas: Different type of foreign bodies such as suture material, talcum powder, etc.
- Granuloma of unknown etiology:
  - Sarcoidosis

Abbreviation: PI3-P, Phosphatidylinositol 3-phosphate

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CHAPTER 4

Molecular Genetics: Basic Principles and Clinical Applications

Chapter Contents

CHROMOSOME

The chromatin of the nucleus is more condensed in the mitotic phase and is known as chromosome. So, chromosome is the coiled and supercoiled deoxyribonucleic acid (DNA) chain along with other proteins within the nucleus. Deoxyribonucleic acid chain takes two complete rounds along the octameric core histone and thus forms a nucleosome. The string of linked nucleosome is helically twisted into a 10 nm fiber, which in turn, is folded into a 30 nm fiber and forms the higher order organization of chromatin.

Chromosome Number

There are total 46 chromosomes in every cell of our body. These 46 chromosomes are arranged in 23 pairs. We get one chromosome of each pair from mother (ovum) and other from the father (sperm). The chromosomes are numbered according to its length from the longest to the shortest. The first 22 pairs of chromosomes are labeled as autosomes, and the last pair is known as sex chromosome. Sex chromosomes consist of either two X chromosomes or one X and one Y chromosome and determine the genetic sex of a person. There are two X chromosomes in a female and one each X and Y chromosome in a male.

Chromosome Structure

Each chromosome has a short arm p (petit) and a long arm q (named as the next letter after p). When chromosomes are arranged for karyotyping, the p arm should always be kept in top (**Box 4.1**). The long and short arms are separated by a constricted point known as centromere (**Fig. 4.1**). As described earlier, the centromere is connected to the mitotic spindle during cell division. Kinetochores are the complexes of proteins that help in the attachment of the centromeres with the mitotic spindle fibers. Kinetochores also help in the verification of the anchoring of the centromeres to the mitotic spindle, activation of the spindle checkpoint, and movement of chromatids during cell division. Each chromosome is protected by the cap of telomere in its two ends. The term telomere name is derived from the Greek word "*telos*" meaning end and "*meros*" meaning part. Telomere is the repetitive nucleotide sequences at the end of the chromosome. At the time of DNA replication, the enzyme DNA polymerase is unable to replicate the DNA sequence of the ends of the chromosomes and the important genetic information may be lost. The telomeres protect the end sequences of DNA, and they themselves become shortened in the process of DNA replication. In each cell cycle, telomere becomes more and more shortened. Therefore, the length of the telomere indicates the age of a cell. In case of absence of telomere, the two ends of the chromosome may fuse together and form a ring.

BOX 4.1 **Human chromosome**

- Total number 46
- Autosomes: 22 pairs
- Sex chromosomes: One pair(X and Y)
- Male: XY, female: XX
- Each chromosome has a short arm p (petit) and a long arm q.
- Centromere: The constricted part between short arm and long arm
- Chromosomal bands are labeled from nearer to far from the centromere
- Telomere
  - The repetitive nucleotide sequences at the end of the chromosome
  - It protects the end sequences of DNA
  - It shortens the process of DNA replication
- Kinetochores
  - Help in the attachment of the centromere with the mitotic spindle fibers
  - Verification of the anchoring of the centromeres to the mitotic spindle

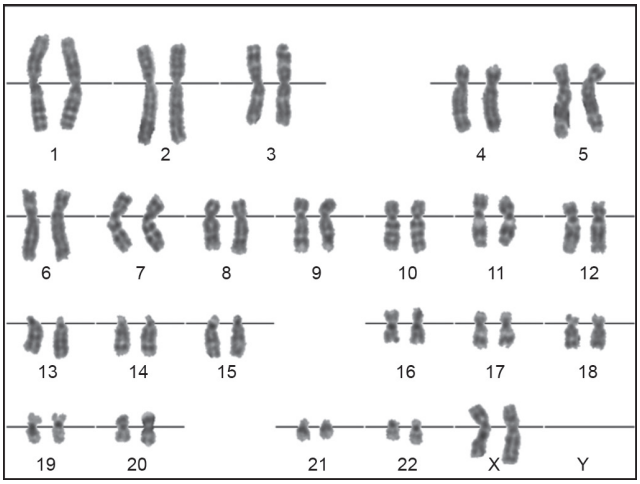

Fig. 4.2: Normal karyotype in a female: 46, XX
Source: Dr Neelam Verma, Professor and Head Department of Hematology, PGIMER, Chandigarh, India

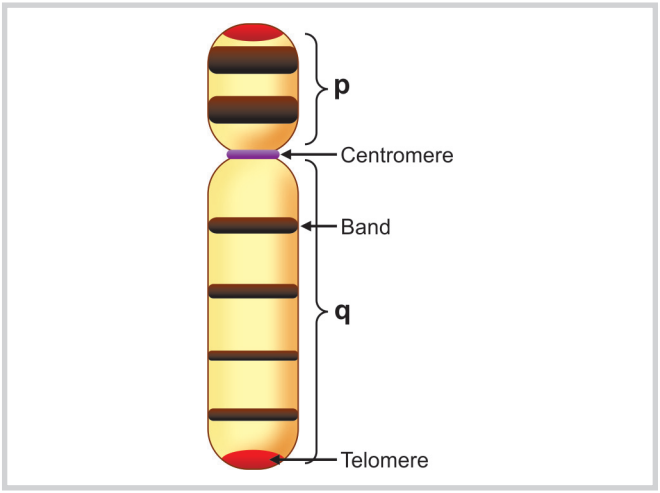

Fig. 4.1: Schematic diagram of a chromosome. Short arm of the chromosome is indicated by "p" and the long arm is represented by "q"

Chromosomes should be stained for visualization under the light microscope. The stained chromosomes look like strings with multiple dark and light horizontal bands. These bands are defined by number: higher the number, the far is the band from the centromere. **Figure 4.2** shows the complete set of human chromosome.

Karyotyping

Karyotype is the study of the number and appearance of chromosome in the cell. It is done by extracting the chromosomes from the blood cell and then the stained chromosomes are seen by light microscope (**Box 4.2**). Photographs of the chromosomes

BOX 4.2 **Chromosomal karyotyping**

Karyotype: The study of the number and appearance of chromosome in the cell

- Chromosomes are extracted and the photographs of chromosomes are taken
- Chromosomes are arranged according to length, band pattern and position of centromeres

Types of banding technique

- G banding
  - Giemsa staining
  - Dark and light band
  - Dark band AT rich, light band GC rich
- C banding
  - Specially stains the constitutive heterochromatin region of the chromosome
- Q banding
  - Stained by fluorescent dye, quinacrine dihydrochloride
  - Visualized by fluorescent microscope
  - Resolution of the bands is better
- R banding
  - Reverse of the G banding
  - Dark region GC, light region AT

Spectral karyotyping

- DNA probes are labeled by five fluorochrome dyes and fluorescence *in situ* hybridization is done
- Used as a screening method for complex chromosomal rearrangements

Digital karyotyping

- Multiple short sequences of DNA are isolated and enumerated.

Abbreviations: AT, adenine-thymine; GC, guanine-cytosine; DNA, deoxyribonucleic acid

are taken and then printed. The chromosomes are arranged according to the following factors:

- Length
- Position of centromeres
- Banding pattern
- Other physical appearances.

The total number of chromosome in the somatic cell is 46 (23 pairs). This is also designated as 2n or diploid. In germ cell, the number of chromosome is 23. This is designated as 'n' or haploid.

There are different types of staining or banding techniques for visualization of chromosomes.

G Banding

Here, the chromosomes are at first treated with trypsin followed by Giemsa staining. Each chromosome is identified by the alternate dark and white band. The dark regions take more stain and are adenine-thymine (AT) rich late-replicating heterochromatin, and light regions are guanine-cytosine (GC) rich early-replicating euchromatin.

Q Banding

The chromosomes are stained by fluorescent dye, quinacrine dihydrochloride. The dark and light bands are visualized under fluorescent microscope. The resolution of the bands is better in this technique.

C Banding

The name C indicates centromeric or constitutive heterochromatin. In this technique, centromere is visualized as stained band. The chromosomes are pretreated with barium hydroxide-saturated solution and followed by Giemsa staining. C banding technique especially stains the constitutive heterochromatin region of the chromosome.

R Banding

It is just the reverse of G banding and dark regions are GC rich and light regions are AT rich.

The other types of techniques, such as spectral karyotyping (SKY) and digital karyotyping can also be used for karyotyping. Digital karyotyping provides quantitative analysis of DNA copy number at high resolution. The multiple short sequences of DNA all over the genome are isolated and enumerated.[1] In SKY, all the chromosomes are stained by chromosome-specific DNA labeled with different fluorochrome.[2]

Chromosomal Abnormalities

There may be two types of chromosomal abnormalities: numerical abnormalities and structural abnormalities (**Box 4.3**):

BOX 4.3 Chromosomal abnormalities

Numerical abnormalities: Gain or loss of whole chromosome
Structural alteration

- Deletion: A segment of chromosome is deleted completely
- Chromosomal duplication: segment of the chromosome is duplicated
- Inversion: Broken part of chromosome is attached upside down
- Translocation
  - Reciprocal translocation: Segments of chromosome from two different nonhomologous chromosomes are broken and then attached to each other reciprocally
  - Robertsonian translocation: One entire acrocentric chromosome attaches with the other chromosome in the centromeric region
  - Ring chromosome: A ring is formed from the broken chromosome

Numerical Abnormalities

Here, a whole chromosome may be missing from the pair, or there may be gain of one more chromosome. In monosomy, there is a loss of one chromosome. This is seen in Turner's syndrome where one X chromosome is less. Gain of one chromosome is seen in Down syndrome. In this disease, there are three copies of chromosome 21.

The common cause of numerical chromosome is nondisjunction of the sister chromatids during migration at the polar ends of the spindle.

Structural Alteration

In case of structural alteration, there is a change of chromosomal DNA arrangement. The chromosomal alteration may be balanced or unbalanced. In balanced alteration, there is no loss or gain of genetic material. However, in unbalanced chromosomal structural alteration, there is either gain or loss of genetic material. This type of unbalanced chromosomal defects is commonly associated with disease.

Deletion

A segment of chromosome is deleted completely, and the impact of deletion depends on the number of the affected genes in that segment of chromosome (**Fig. 4.3**).

Chromosomal Duplication

Here the particular segment of the chromosome is duplicated, and therefore, there are three copies of particular segment of DNA within the nucleus.

Inversion

A segment of chromosome is broken and again attached in the chromosome with upside down (**Fig. 4.3**).

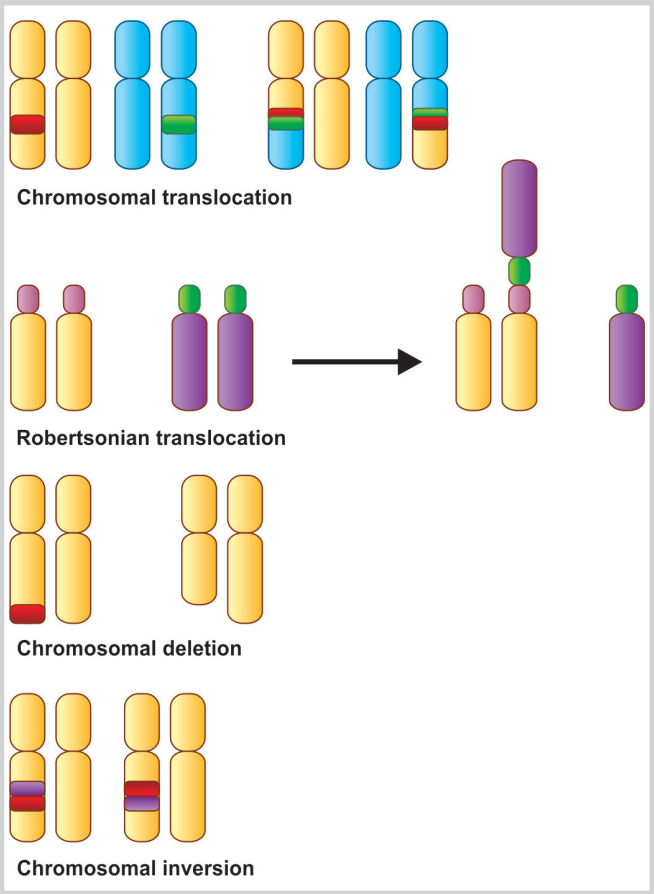

Fig. 4.3: Schematic diagram showing different types of chromosomal changes

Translocation

In case of translocation, a segment of chromosome is broken and then transferred to any other chromosome. There are two types of translocation: reciprocal translocation and Robertsonian translocation. In case of reciprocal translocation, the segments of chromosomes are broken from two different nonhomologous chromosomes and then attached to each other reciprocally. Therefore, the total amount of genetic material remains constant. In Robertsonian translocation, one entire chromosome is fused with another chromosome in the centromeres region to form a single chromosome (**Fig. 4.3**).

Ring

A segment of chromosome is broken and forms a circle of ring.

Chromosomal Abnormalities in Malignancies

Cancer is believed to develop from progressive series of multiple genetic abnormalities in a clone of cells.[3] To date, cytogenetic abnormalities have been noted in large number of patients with cancer.[4] The demonstration of cytogenetic abnormalities has emerged as prognostic and predictive markers in hematologic cancers and certain types of solid tumors. Moreover, the knowledge on cytogenetic abnormalities has provided us insights into the mechanisms of carcinogenesis (**Box 4.4**).

The chromosomal changes in cancer may be due to (1) primary chromosomal changes that are mainly responsible for the initiation of the tumor and (2) secondary or tumor-associated changes that are associated with tumor growth and progression.

BOX 4.4 Chromosomal changes in malignancies

Chromosomal rearrangement
- Chimeric fusion gene with new activity
  - Aberrant or enhance tyrosine kinase activity: Philadelphia chromosome in chronic myeloid leukemia t(9;22)(q34.1;q11.23)
  - Aberrant or enhanced transcriptional activity: Acute promyelocytic leukemia t(8;21)(q22;q22.3)
- Enhance the activity of the proto-oncogene: juxtaposition of the proto-oncogene coding sequence near a normal tissue regulatory element may enhance the activity of the proto-oncogene
- Chromosomal gain or loss
  - Chromosomal gain
    - Complete or partial trisomy
    - Intrachromosomal amplification of genes
    - Extrachromosomal amplification of genes
    - Certain gene activity enhances leading to tumor initiation or progression
  - Chromosomal loss: Portion of gene is lost

Chromosomal Rearrangement

Chromosomal rearrangement occurs in reciprocal translocation, inversion and insertion. Reciprocal translocation is the characteristic cytogenetic hallmark of leukemia and lymphoma. Specific chromosomal translocation of breakpoint cluster region-ABL1 (BCR-ABL1) fusion gene acts as sensitive indicators in the assessment of response to chemotherapy in chronic myeloid leukemia (CML).[5] Due to chromosomal rearrangement, two things may happen: (1) a chimeric fusion gene with new activity and (2) chromosomal changes leading to excessive expression of normal gene.

Chimeric Fusion Gene

The majority of the chromosomal rearrangement causes fusion of two genes and therefore produces a chimeric gene. These chimeric genes may produce (**Fig. 4.4**):
- Aberrant or enhanced tyrosine kinase or
- Aberrant or enhanced transcriptional activity.

Aberrant or Enhanced Tyrosine Kinase

The best example of reciprocal translocation involving tyrosine kinase gene is Philadelphia chromosome in CML. It is the result of a reciprocal translocation, t(9;22)(q34.1;q11.23)

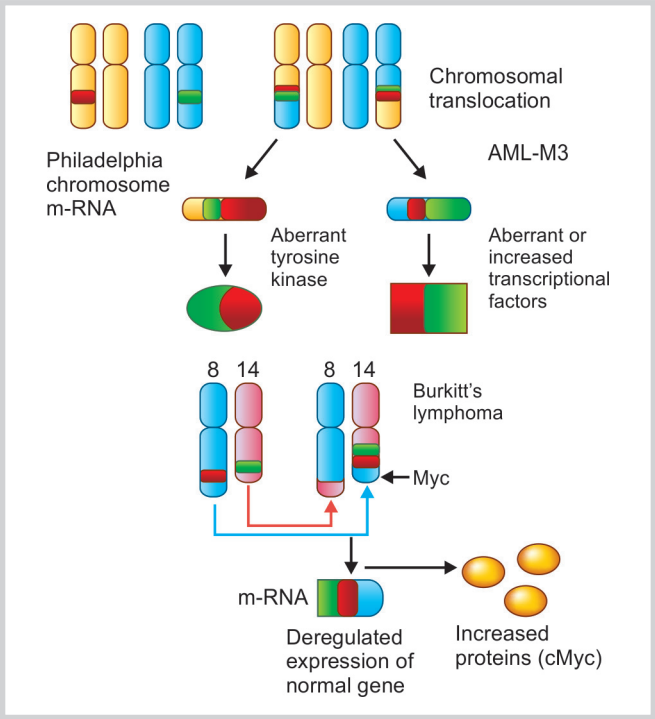

Fig. 4.4: Schematic diagram showing effects of chromosomal translocations in tumor development

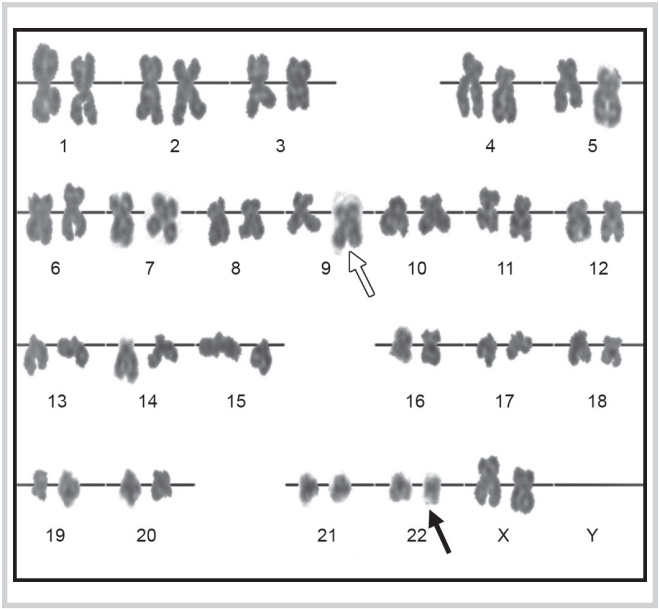

Fig. 4.5: Philadelphia chromosome in a female patient with chronic myelogenous leukemia: 46, XX, t(9;22)
Source: Dr Neelam Verma, Professor and Head, Department of Hematology, PGIMER, Chandigarh, India

(**Fig. 4.5**). The BCR gene on band 22q11.23 is joined to the portions of the gene encoding the cytoplasmic ABL1 tyrosine kinase on band 9q34.1. This causes production of a chimeric protein, BCR-ABL1 that has aberrant tyrosine kinase activity.[6] The chimeric tyrosine kinase gene formation has been noted in various other malignancies such as non-small cell lung carcinoma, anaplastic large cell lymphoma, multiple myeloma, acute lymphoblastic leukemia and papillary thyroid carcinoma (**Table 4.1**).

Aberrant or Enhanced Transcriptional Activity

Chromosomal translocation may generate chimeric fusion gene that produces aberrant or increased transcription factors (**Table 4.1**). In case of acute promyelocytic leukemia, there is t(8;21)(q22;q22.3). This produces a fusion gene RUNX1-RUNX1T1. This chimeric gene generates a transcription factor. The binding of the chimeric transcription factors with its target gene inhibits normal myeloid differentiation and contributes to the accumulation of immature myeloid cells in acute myeloid leukemia.[7]

Deregulation of Normal Gene

Chromosomal translocation may help juxtapose the proto-oncogene coding sequence near a normal tissue regulatory element and may enhance the activity of the proto-oncogene. In Burkitt's lymphoma, there is translocation of t(8;14)(q24.21;q32.33) that causes juxtaposition of c-MYC and immunoglobulin heavy chain gene leading to deregulation of c-MYC transcription (**Fig. 4.4**).[8]

Chromosomal Gain or Loss

Chromosomal loss may occur due to deletion of the segment of gene (**Fig. 4.6**). This deletion of genetic material causes initiation or progression of malignancies (**Table 4.1**). The chromosomal deletions may be specific to a particular tumor, such as 11p13 deletions are restricted to Wilms' tumor and del(13)(q14q14) deletion is observed only in retinoblastoma. Gene deletion may occur in multiple chromosomes of a particular cancer. Therefore, sometimes it is difficult to ascertain what exact gene or genes are responsible for tumorigenesis.

In case of chromosomal gain, certain gene activity enhances leading to tumor initiation or progression. Genomic gains commonly occur due to complete or partial trisomy, intrachromosomal and extrachromosomal amplification of genes. In large scale, genomic gain there is gain of large segment of chromosome affecting multiple genes. Trisomy of chromosome number 8 is the example of large genomic gain that is commonly seen in myelodysplastic syndrome, myeloproliferative disease, acute myeloid leukemia and acute lymphoblastic leukemia.

TABLE 4.1: Chromosomal translocation in hematological and lymphoid malignancies

Disease	*Rearrangement*	*Gene involved*
Gene fusion		
Chronic myeloid leukemia, acute lymphoblastic leukemia, acute myeloid leukemia	t(9;22)(q34.1;q11.23)	BCR-ABL1
Anaplastic large cell lymphoma	t(2;5)(p23;q35)	ALK-NPM1
Multiple myeloma	t(4;14)(p16.3;q32.33)	WHSC1-IGHG1
Acute myeloid leukemia	t(8;21)(q22;q22.3)	RUNX1-RUNX1T1
Acute myeloid leukemia	t(9;11)(p22;q23)	MLL-MLLT3
Acute megakaryoblastic leukemia	t(1;22)(p13;q13)	RBM15-MKL1
Acute promyelocytic leukemia	t(15;17)(q22;q21)	PML-RARA
Burkitt's lymphoma	t(8;14)(q24.21;q32.33)	MYC-IGHG1
Follicular lymphoma	t(14;18)(q32.33;q21.3)	IGHG1-BCL2
Mantle cell lymphoma	t(11;14)(q13;q32.33)	CCND1-IGHG1
Gene nonfusion		
T-Acute lymphoblastic leukemia	t(8;22)(q24;q11)	c-MYC(8q24)
B-CLL	t(8;12)(q24;q22)	c-MYC(8q24)
T-ALL	t(7;19)(q35;p13)	LYL1(19p13)
DLBCL	t(3;14)(q27;q32)	Laz3/BCL-6(3q27)
B-NHL	t(10;14)(q24;q32)	Lyt-10(10q24)

Abbreviations: DLBCL, diffuse large B-cell lymphomas; BCLL, B-cell chronic lymphocytic leukemia; T-ALL, T-cell acute lymphoblastic leukemia; B-NHL, B-cell non-Hodgkin lymphomas; PML-RARA, progressive multifocal leukoencephalopathy-retinoic acid receptor alpha

In case of solid tumors, trisomy of chromosome 7 is noted in malignant neurogenic tumor.[9]

Gains affecting small genomic regions or single genes amplification are infrequent. Homogeneously staining region (HSR) indicates focal amplification of small number of genes (**Fig. 4.6**).[10]

The HSR does not show any typical banding pattern. Gene amplification is specifically seen in some solid tumors with poorer prognosis.[11] Extrachromosomal DNA amplification is seen as dmin (**Fig. 4.6**). These are circular in shape and autonomously replicating DNA strands of variable size.

Modern technologies such as comparative genomic hybridization (CGH) and single-nucleotide polymorphism genotyping may be helpful to detect such single gene amplification.[12]

MOLECULAR CYTOGENETIC TECHNIQUES

In the last few decades, there is massive development of cytogenetic techniques. These techniques are more sophisticated, sensitive and can be done on archival material bypassing the cell culture. The comparison of different techniques has been done in **Table 4.2**.

Fluorescent Hybridization Technique

Fluorescence *in situ* hybridization (FISH) uses specific DNA probes labeled with fluorescent dyes for the detection of genetic changes in the chromosome of the individual cell

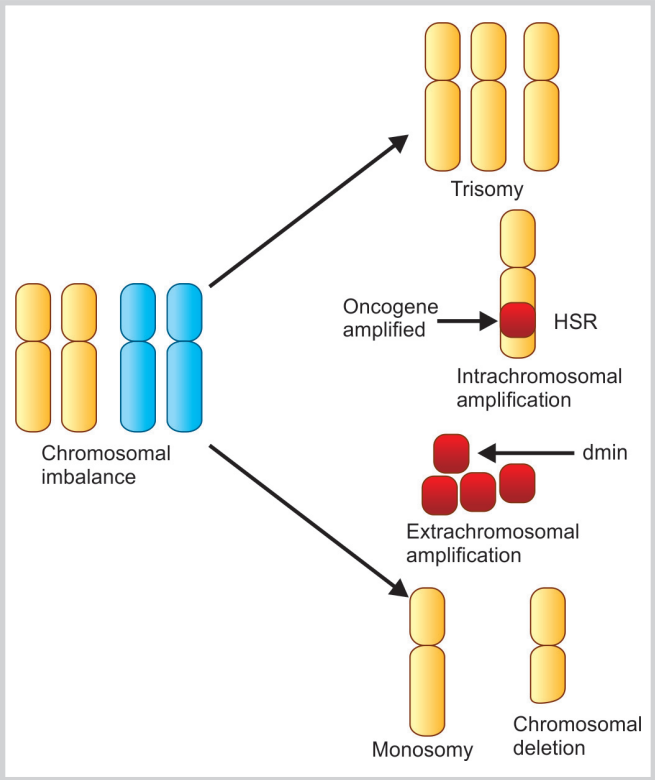

Fig. 4.6: Schematic diagram showing effects of chromosomal imbalance in tumorigenesis

level. Fluorescence *in situ* hybridization is also known as "interphase cytogenetics". This technique can be performed in the cytology as well as paraffin embedded histology sections.

Principle

The principles of FISH are (**Fig. 4.7**):

- The double-helix DNA is made into single-stranded DNA by heat denaturation
- A suitable complementary fluorescent dye labeled DNA probe is used for target DNA
- The DNA-specific complementary probe binds with the target DNA and forms a new DNA duplex
- The hybridized DNA probe with target DNA is then visualized by fluorochrome dye.

Advantages of Fluorescence In Situ Hybridization

Fluorescence *in situ* hybridization technique has several advantages such as:

- It has high resolution capability
- It helps to demonstrate the specific location of DNA sequence directly in the chromosome. Fluorescence *in situ* hybridization is helpful to correlate the cytogenetic alteration along with morphological detail

TABLE 4.2: Comparison of different cytogenetic technique

Technique	*Basic principles*	*Advantages*	*Limitations*	*Comments*
Traditional cytogenetic by band technique	Cells are cultured, and chromosome is stained with dye	• Easy and simple • Low cost • Robust	• Poor resolution • Cell culture is needed	It is a traditional old technique to detect structural and numerical chromosomal abnormalities
FISH technique	The double helix DNA is made into single-stranded DNA and a probe of complementary DNA is hybridized with the target DNA segment and visualized	• High resolution • It can be done in interphase cell and do not need cell culture • Archival material can be used • It correlates the cytogenetic alteration to morphological detail	• Only the known abnormality is detected with selected available probes • As cytogenetic data of currently used probe is only available so it is not a good screening test	Chromosomal translocation and amplification is well-visualized
CGH	Tumor DNA and normal reference DNA is differentially labeled and then hybridized with metaphase spread of chromosome. The relative fluorescence level of tumor and normal DNA is measured	• Good screening test • Genome-wise screening is possible	• Low resolution • Balanced rearrangement is undetected • Exact structural change in the chromosomal gain or loss	Chromosomal amplification and deletion can be demonstrated

Abbreviations: DNA, deoxyribonucleic acid; CGH, comparative genomic hybridization

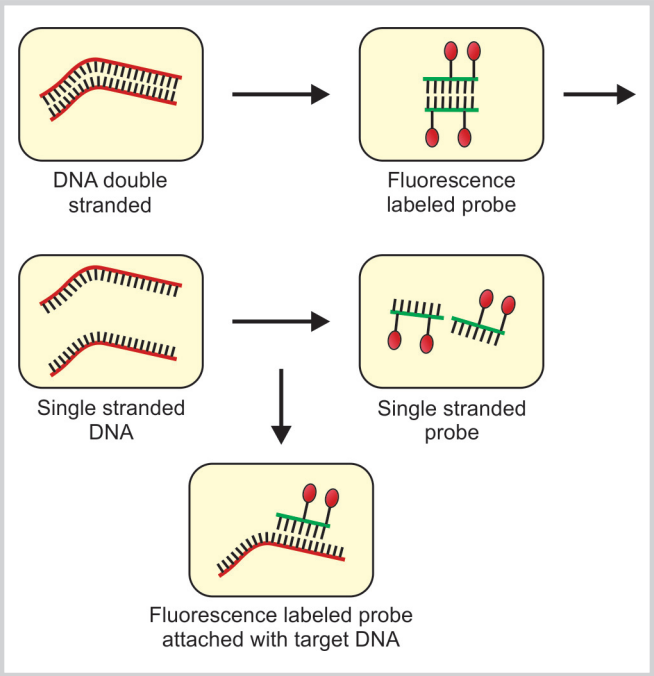

Fig. 4.7: Basic principles of fluorescence *in situ* hybridization technique are highlighted in the schematic diagram

- It can be done on archival material
- No cell culture technique is needed for FISH technique, and it can be done in interphase state of cells. Therefore, FISH is greatly helpful in cytogenetic of solid tumors
- Fluorescent tags are safer, simpler and easy to store for indefinite period of time.

Probes Used in Fluorescence In Situ Hybridization

Various types of DNA probes are used for FISH.

Centromeric Probes

These types of probes are made up of alpha satellite DNA and are directed against highly repetitive sequences in the centromeric region of the chromosome.[13] They give very strong detectable signals. These probes are generally used for gain or loss of chromosome.

Telomeric Probes

These probes are designed to target the repeated sequences in the telomeric region at the long or short arm of chromosome. In interphase cells, the terminal deletion of chromosomal segment at the telomeric region can be detected by telomeric probe.

Chromosome Painting

The whole chromosome probe is used in case of chromosome painting. It is used to demonstrate chromosomal translocation in the interphase cells.[14]

Probes against Unique Sequence

Here, DNA probes are used against single copy gene. This type of unique sequence probes are used for the detection of chromosomal translocation, deletion, and gene amplification.[15]

Multicolor Fluorescence *In Situ* Hybridization

In multicolor FISH (M-FISH), multiple fluorochrome dyes are used in combination along with different filter band for visualization of color in the fluorescent microscope. Using five to six types of fluorochrome dyes by combinatorial or ratio labeling technique, it is possible to visualize simultaneously all the human chromosome, each one in different color in a single hybridization.

Spectral Karyotyping

In case of SKY, DNA probes are labeled by five fluorochrome dyes in combination for FISH. The hybridization is presented in both display and classification colors. On the basis of spectral measurements at each pixel of the image, a chromosome classification algorithm is used to spectrally karyotype all human chromosomes.[2]

The SKY is used as screening method for complex chromosomal rearrangements.

Comparative Genomic Hybridization

Basic principles of CGH (**Fig. 4.8**) are:

- Deoxyribonucleic acid is extracted both from test sample and normal reference sample
- Biotinylated total tumor DNA and digoxigenin-labeled normal genomic reference DNA are simultaneously hybridized to normal metaphasic DNA
- Tumor or test DNA and normal DNA is differentially labeled. Hybridization of tumor DNA is detected with green-fluorescing fluorescein isothiocyanate-avidin, and the reference DNA hybridization is detected with red-fluorescing rhodamine antidigoxigenin
- The relative amount of tumor and normal reference DNA bound at a given locus of the metaphase chromosome will depend on the relative abundance of those sequences in the test or reference sample. If a region of DNA is amplified in the test DNA then the corresponding region of the metaphase chromosome will be predominantly green color. Conversely, if a region of DNA is deleted then the corresponding region of the metaphase chromosome will be predominantly red color
- The ratio of green-to-red fluorescence (test to reference fluorescence) is measured with the help of image analyzer
- Increased green fluorescence indicates gain or amplification of DNA, whereas increased red fluorescence indicates deletion of DNA.

Advantages of comparative genomic hybridization

- Comparative genomic hybridization can be used in a single cell

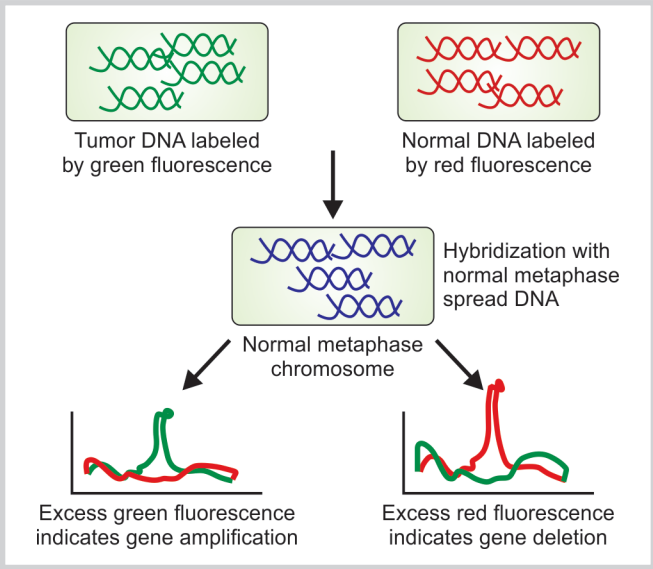

Fig. 4.8: Schematic diagram showing basic principles of comparative genomic hybridization

- This technique does not require any prior knowledge of the chromosome imbalance that is involved in tumor DNA
- It can be done on a small amount of microdissected cells.

Disadvantages

- Comparative genomic hybridization has limited use if the chromosomal rearrangement does not cause any genomic imbalance
- It does not give any information about the exact structural change in the chromosomal gain or loss.

Three-dimensional Fluorescence *In Situ* Hybridization

In case of three-dimensional FISH (3D FISH), the nuclei are fixed and the chromosomal territories are well-preserved. With the help of suitable image acquisition technology, the series of images collected from the multiple planes of the nucleus are reconstructed and a 3D image is made. Three-dimensional FISH helps to study the higher order chromatin structure.[16]

Living Cell Cytogenetics (Four dimensional Fluorescence *In Situ* Hybridization)

Incorporation of fluorescent nucleotides into DNA during replication in S phase helps in the visualization of DNA and proteins in the living cells.[17] The growth and division of the labeled cells help in the visualization of the genomic organization in the subsequent generations.

Clinical Applications of Cytogenetics

The old traditional and newer molecular cytogenetic has great impact on diagnosis, screening of diseases, and prognostic assessment of cancer.

Diagnosis: The demonstration of specific chromosomal abnormalities is one of the diagnostic features of many hematological malignancies. The cytomorphology along with flow cytometric findings and molecular cytogenetic are essential diagnostic features of subclassification of lymphomas such as in diffuse large B-cell lymphomas and follicular lymphoma (FL).[18] The detection of t(14;18) is a diagnostic marker of FL. The detection of reciprocal t(11;14) by FISH is essential in diagnosing and distinguishing mantle cell lymphoma from other lymphomas.[19] Demonstration of Philadelphia chromosome [chromosomal translocation of t(9;22)] is the important diagnostic marker of CML. Acute myelogenous leukemia-M3 is characterized by t(15;17) (**Fig. 4.9**).

Chromosomal abnormalities are also specific for diagnosis of many solid tumors such as Ewing's sarcoma, retinoblastoma, synovial sarcoma, etc. (**Tables 4.3** and **4.4**).

PROGNOSIS OF THE PATIENT

Certain chromosomal abnormalities are related with prognosis of the patients, such as amplification of c-erb-B-2 is related with bad prognosis of breast cancer. Gains of chromosomes 7 and 8 are associated with poor prognosis and potential markers for tumor aggressiveness in prostate cancer.[20]

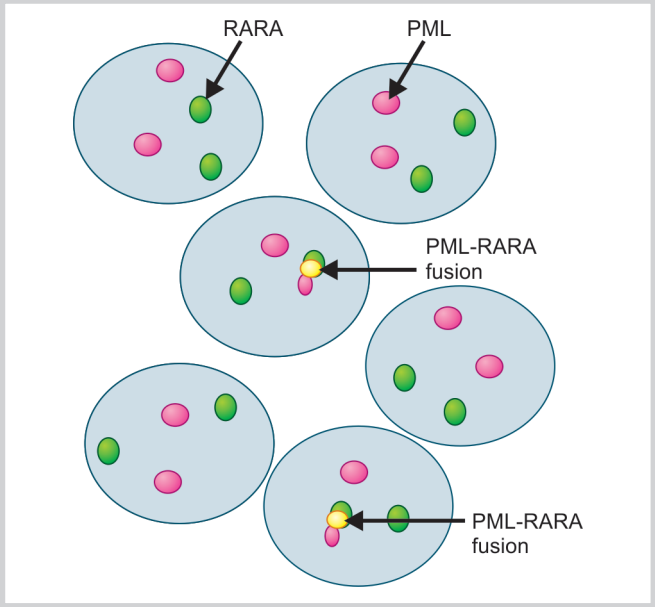

Fig. 4.9: Schematic diagram showing progressive multifocal leukoencephalopathy (red) and retinoic acid receptor, alpha (green) gene and chromosomal translocation (yellow) in case of acute promyelocytic leukemia

TABLE 4.3: Chromosomal translocation in solid cancer

Disease	*Rearrangement*	*Gene involved*
Gene fusion		
Non-small cell-lung cancer	inv(2)(p22-p21p23)	EML4-ALK
Papillary thyroid cancer	inv(10)(q11.2q11.2)	RET-NCOA4
Papillary thyroid cancer	inv(10)(q11.2q21)	RET-CCDC6
Follicular thyroid cancer	t(2;3)(q12-q14;p25)	PAX8-PPARG
Ewing's sarcoma	t(11;22)(q24.1-q24.3;q12.2)	FLI1-EWSR1
Ewing's sarcoma	t(21;22)(q22.3;q12.2)	ERG-EWSR1
Synovial sarcoma	t(x;18)(p11.2;q11.2)	Not identified
Melanoma of soft parts	t(12;22)(q13;q12)	ATF 1(12q13) EWS(22q12)
Liposarcoma	t(12;16)(q13;p11)	CHOP(12q13) FUS(16p11)
Rhabdomyosarcoma	t(2;13)(q35;q14)	PAX3(2q35) FKHR(13q14)

Abbreviations: ERG-EWSR1, Ets related Gene-Ewing sarcoma breakpoint region 1; ATF 1, Activating transcription factor 1; EWS, Ewing sarcoma; FUS, fused in sarcoma; CHOP, cyclophosphamide + doxorubicin + vincristine + prednisolone; FKHR, forkhead in human rhabdomyosarcoma

Recurrence of Malignancy

The demonstration of certain chromosomal abnormalities indicates early recurrence, such as in:

- Bladder cancer
- Detection of malignancy in cerebrospinal fluid.

Monitoring Therapy

The demonstration of the cytogenetic markers during the time of chemotherapy or after treatment may be helpful to monitor the response of the treatment. Significant reduction of Philadelphia (Ph) chromosome in CML patients indicates sustained chronic phase and better survival. Therefore, the demonstration of Ph chromosome by cytogenetics is a valuable tool for monitoring response to interferon in CML therapy.

Monitoring of Surgical Margins in Head and Neck Carcinomas

The detection of chromosomal markers in the surgical margins of the head and neck cancer patient may be helpful to assess the tumor-free resection margin.[21]

TABLE 4.4: Chromosomal deletion and gain in solid tumors

Tumor	*Deleted chromosome*	*Gene involved*
Chromosomal deletion		
Neuroblastoma, medullary thyroid carcinoma, pheochromocytoma, MEN-2, melanoma	1P	ND
Breast carcinoma	1p,2q	ND
Leiomyosarcoma	3q	ND
Anaplastic oligodendroglioma	19q	ND
Melanoma	7q	ND
Colorectal carcinoma	17p, 18q, 11p13	ND
MEN-1	11q	ND
Renal-cell cancer	del(3p26-p25)	VHL gene involved
Colon cancer	del(4)(q12)	REST gene involved
Colon cancer	del(5)(q21-q22)	APC
Retinoblastoma	del(13)(q14.2)	RB1
Wilms' tumor	del(X)(q11.1)	FAM123B
Testicular tumors	12q	ND
Uterine cancers	del(10)(q23.3)	PTEN
Chromosomal gain by amplification		
Breast cancer	amp(1)(q32.1)	IKBKE
Breast cancer	amp(6)(q25.1)	ESR1
Neuroblastoma	amp(2)(p24.1)	MYCN
Hepatocellular carcinoma	amp(11)(q13-q22)	YAP1, BIRC2
Malignant melanoma	amp(3)(p14.2-p14.1)	MITF
Nonsmall cell-lung cancer	amp(14)(q13)	NKX2-1

Abbreviations: ND, not detected; MEN-2, multiple endocrine neoplasia, type 2; VHL, von Hippel-Lindau; APC, adenomatous polyposis coli; Rb1, retinoblastoma 1; IKBKE, inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon; MYCN, myelocytomatosis viral related oncogene; MITF, microphthalmia-associated transcription factor

CONCLUSION

Recently, due to the massive advancement of techniques in interphase cytogenetics, it is possible to demonstrate various cytogenetic aberrations in cytology material and paraffin embedded sections. The FISH technique helps in the diagnosis of different hematological and solid tumors easily and reliably. It is now also possible to predict early recurrence, monitoring therapy and planning the target therapy on the basis of cytogenetic. The various commercially available probes can be used in cytology samples reliably and effectively. Presently, CGH is used to screen quantitative genomic alterations in various cancers. This technique is promising and in conjunction with other ancillary tools such as immunocytochemistry and flow cytometry it may be helpful in detection and classification of cancer in cytology sample.

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CHAPTER 5

Neoplasm

Chapter Contents

INTRODUCTION

The term neoplasm means new growth. The word "neoplasm" is originated from the Greek word "*neo*" means new and "*plasm*" means formation. The term "tumor" is originally derived from the Latin word for swelling. However, presently the word tumor is now used as synonymous to neoplasm. It is difficult to exactly define neoplasm. The British oncologist Willis defined neoplasm as: "A neoplasm is an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with that of the normal tissues, and persists in the same excessive manner after cessation of the stimulus which evoked the change".[1] This definition is true for most of the neoplasm in our body.

Neoplasm can be divided into two types: benign and malignant.

BENIGN NEOPLASM

Benign tumors have certain important characteristics:

- They have restricted growth rate
- They are usually well-encapsulated and not invasive to the surrounding tissue
- They are well-differentiated
- They do not metastasize.

Benign tumors are usually classified on the basis of their cells of origin. Tumors arising from the specific type of tissue resemble their cell of origin, such as a tumor that arises from the gland will show glandular differentiation. In general, the suffix "oma" is attached to the cell of origin of benign tumors. For example, the tumor of fat is known as "lipoma"; tumor of osteoblast is known as osteoma, tumor of chondroid element is known as "chondroma". Tumor of the endodermal glandular epithelium is known as adenoma, whereas the tumor of epithelial origin is known as papilloma (**Table 5.1**).

A malignant tumor is rapidly growing and aggressive in nature. They often show poor resemblance with its cell of origin, locally invasive and metastasize to the distant organ. Cancer is a common term for all types of malignant tumor. The word "cancer" probably came from Latin which means crab. This is because the cancer in any tissue clings with it firmly.

The nomenclature of malignant tumor is almost same as that of benign tumor. Malignant tumors originating from the epithelial cell are labeled as carcinoma. Carcinoma is further classified depending on the precise cell of origin, such as carcinoma developed from the glandular epithelium labeled as adenocarcinoma (**Figs 5.1 and 5.2**) whereas, the carcinoma developed from the squamous epithelium is labeled as squamous

TABLE 5.1: Classification of neoplasm

Tissue of origin	*Benign*	*Malignant*
Epithelial tissue		
Stratified squamous,basal cells of skin or adnexa	Squamous cell papilloma	Squamous cell carcinoma
Columnar epithelium	Adenoma	Adenocarcinoma
Respiratory epithelium	Bronchial adenoma	Bronchogenic carcinoma
Renal epithelium	Renal adenoma	Renal cell carcinoma
Urinary tract epithelium	Transitional cell papilloma	Transitional call carcinoma
Mesenchymal tissue		
Blood vessels and lymphatic tissue	Hemangioma Lymphangioma	Angiosarcoma, Kaposi's sarcoma
Mesothelial cells	Mesothelioma	Malignant mesothelioma
Fat	Lipoma	Liposarcoma
Striated muscle	Rhabdomyoma	Rhabdomyosarcoma
Smooth muscle	Leiomyoma	Leiomyosarcoma
Fibrous tissue	Fibroma	Fibrosarcoma
Bone	Osteoma	Osteosarcoma
Cartilage	Chondroma	Chondrosarcoma
Trophoblast	Hydatidiform mole	Choriocarcinoma
Germ cell	Mature teratoma	Malignant teratoma, seminoma, dysgerminoma

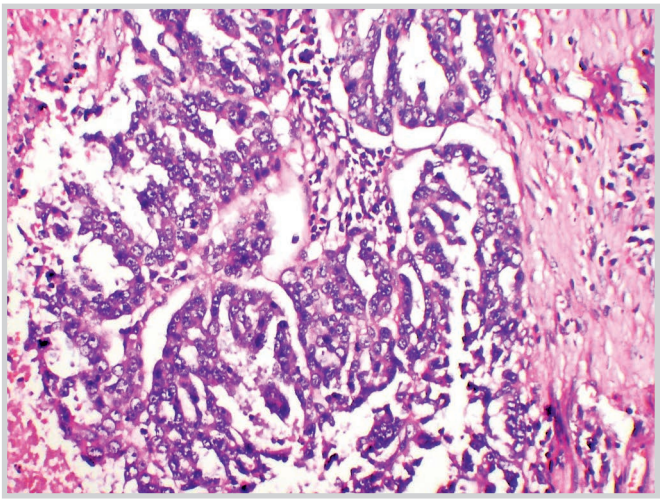


Fig. 5.1: Histology section of an adenocarcinoma of uterus (hematoxylin and eosin X LP)

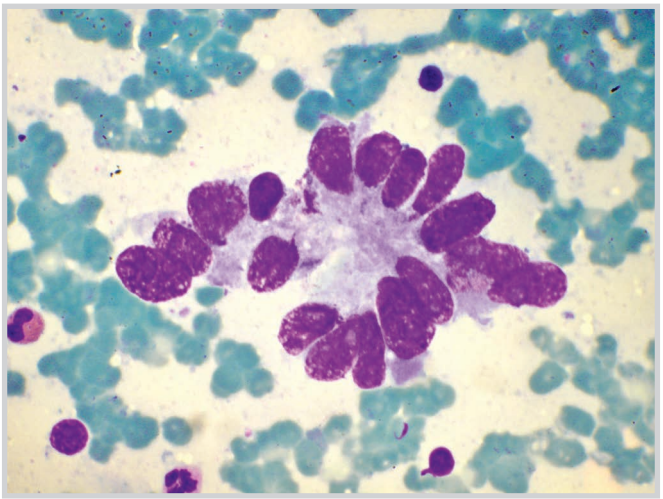


Fig. 5.2: Cytology smear of an adenocarcinoma showing gland like arrangement of malignant cells (May Grunwald Giemsa X MP)

cell carcinoma (**Figs 5.3** and **5.4**) (**Table 5.1**). Malignant tumors arising from mesenchymal tissues are known as sarcoma (**Fig. 5.5**). For example, malignant tumor of bone, fibrous tissue, or fat cells are labeled as osteosarcoma, fibrosarcoma, and liposarcoma, respectively.

The tumor may undergo divergent differentiation of the parenchymal tissue into mesenchymal tissue, and this is known as a mixed tumor, such as pleomorphic adenoma of salivary gland or adenofibroma of the uterus. The certain tumors develop from totipotent germ cells and are labeled as teratoma. The teratoma may show different types of epithelial and mesenchymal tissue simultaneously, such as mature cystic teratoma of the ovary often shows squamous epithelium, glands, sebaceous cells, cartilage, or bone (**Fig. 5.6**). Tumors developed from germ cells are known as dysgerminoma/seminoma (**Fig. 5.7**).

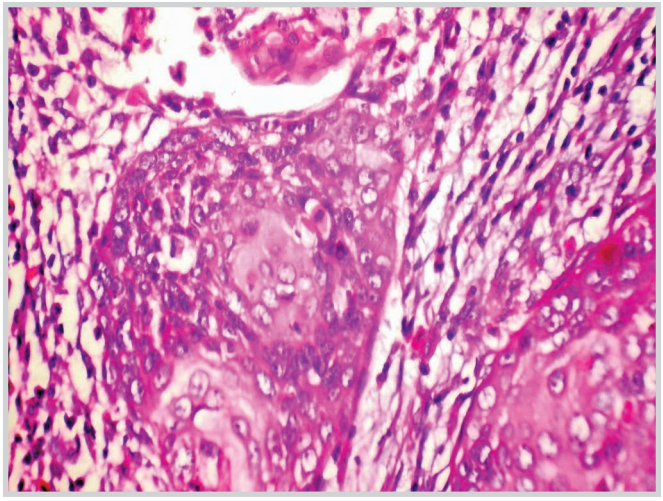


Fig. 5.3: Histology section of squamous cell carcinoma of cervix (hematoxylin and eosin X LP)

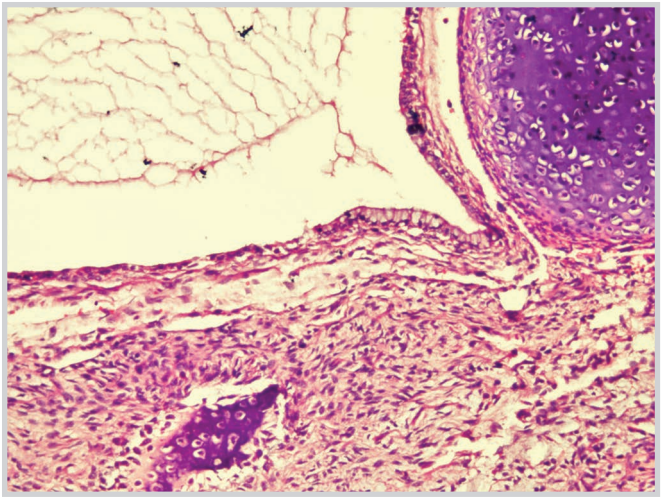


Fig. 5.6: Histology section of mature cystic teratoma of ovary showing cartilage, gland and adipose tissue (hematoxylin and eosin X LP)

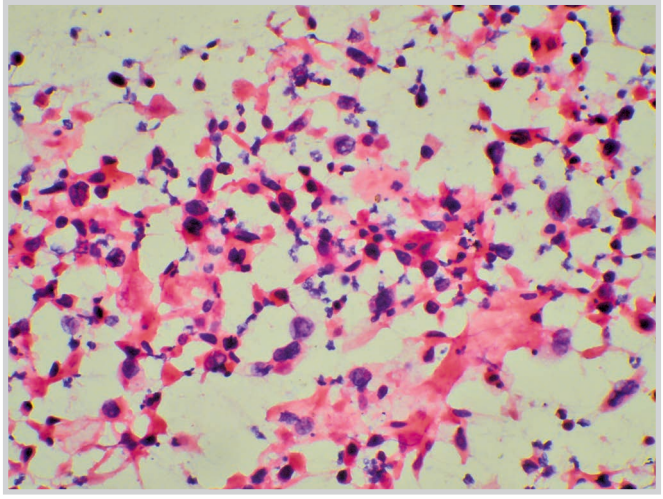


Fig. 5.4: Cytology smear of a squamous cell carcinoma shows oval to polyhedral cells with hyperchromatic nuclei (hematoxylin and eosin X MP)

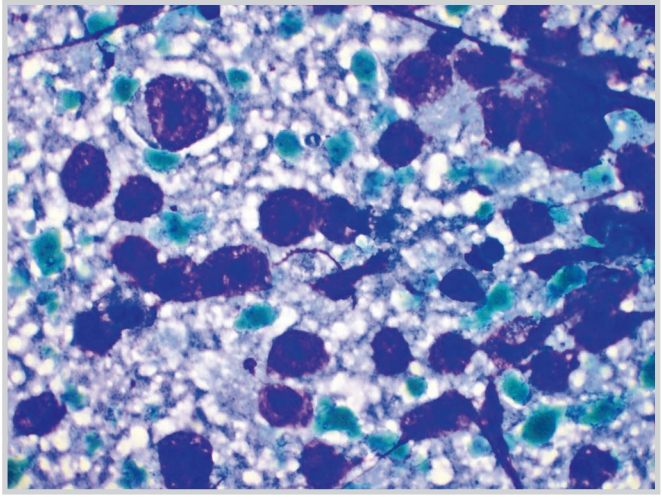


Fig. 5.7: Cytology smear of a seminoma of testis showing immature germ cell in a foamy vacuolated background (May Grunwald Giemsa X MP)

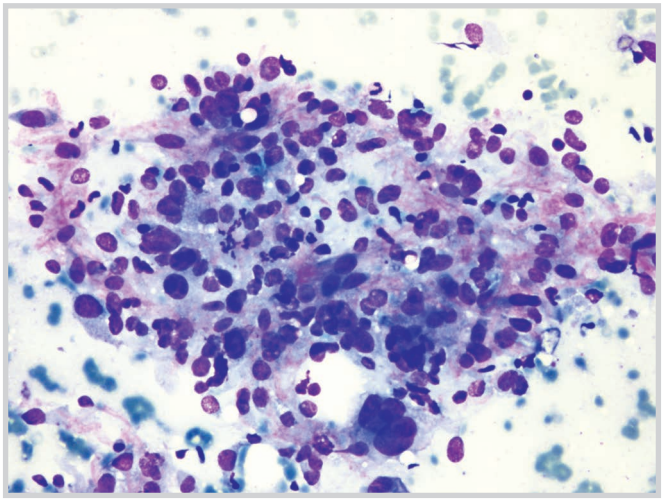


Fig. 5.5: Cytology smear of a sarcoma shows oval to elongated cells with marked nuclear pleomorphism (May Grunwald Giemsa X MP)

Misnomers

The suffix "oma" is attached with the name of some tumors, and they wrongly give an impression of benign tumors, such as: seminoma: a malignant germ cell tumor of testis; hepatoma: malignant tumor of heptocytes, and lymphoma: malignant tumor of the lymphoid cells, etc.

BIOLOGICAL CHARACTERISTICS OF MALIGNANT TUMOR OR CANCER

A malignant tumor is always aggressive in behavior and sooner or later kills its host. The principal differences of a benign and malignant neoplasm are highlighted in the **Table 5.2**.

HALLMARKS OF CANCER

There are certain important hallmarks of cancer.[2] These are as follows (**Fig. 5.8**):

TABLE 5.2: Differentiating features of benign and malignant tumor

Feature	*Benign*	*Malignant*
Growth	Slow	Rapid
Proliferative activity	Low mitotic rate	High mitotic rate and low cell death or apoptosis
Capsule	Usually well encapsulated	Unencapsulated, poorly-defined border
Local invasion	Absent	Locally invasive
Metastasis	Absent	Distant metastasis
Differentiation	Resembles the tissue of origin	Poor differentiation
Behavior	Modest, usually does not kill the patient	Aggressive and ultimately kills its host

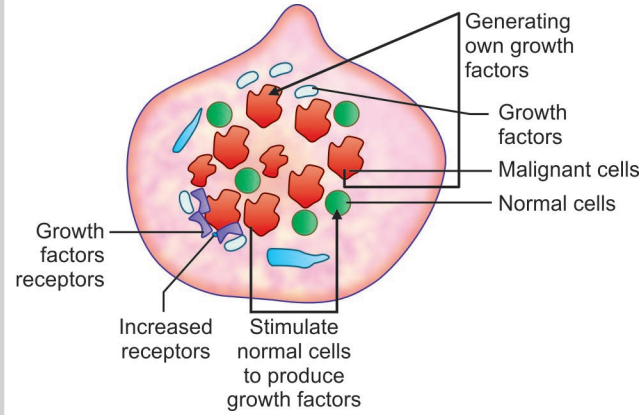


Fig. 5.9: Schematic diagram shows mechanisms of sustained proliferation of cells in cancer. Tumor cells generate their own growth factor ligands, stimulate the tumor-associated normal cells for production of growth factor, and alter the intracellular pathways of receptors

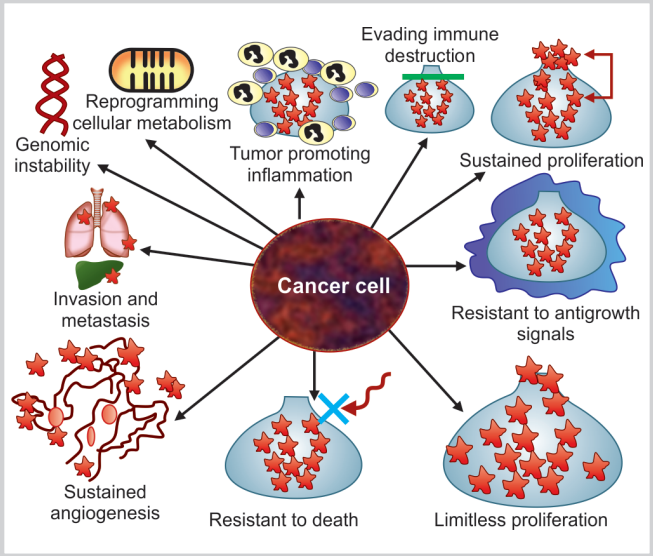


Fig. 5.8: Schematic diagram shows various hallmarks of cancer

- Sustained proliferation without any growth signal
- Insensitivity to antigrowth signal (evading growth suppressor)
- Resisting cell death or evading apoptosis
- Limitless replicative potential
- Sustained angiogenesis
- Tissue invasion and metastasis
- Reprogramming cellular metabolism
- Genomic instability and mutation
- Tumor promoting inflammation
- Evading immune destruction.

Sustained Proliferation

The cancer cells have the great capability to grow or proliferate by their own without any exogenous growth stimulation. They can achieve this by:

- Generating own growth factor ligands
- Stimulating the tumor-associated normal cells for the production of growth factor[3]
- Altering the cell-surface receptors or intracellular receptor pathways (**Fig. 5.9**).[4]

Insensitivity to Antigrowth Signal (Evading Growth Suppressor)

Cancer cells acquire the immense capability to overcome the antigrowth signals that normally operate in the system to maintain tissue homeostasis and cellular quiescence.

The evasion from growth suppression can be done by (**Fig. 5.10**):

- Disruption of pRb and p53 pathways: Cancer cell continuously recruits the cells in the cell cycle by disruption of pRb and p53 pathway due to mutation of Rb and p53 gene. Functional activity of pRb may also be lost due to sequestration of pRb by viral oncoprotein (such as E7)[5]
- Disruption of transforming growth factor beta pathway that originally blocks pRb activity: This may occur by mutation, dysfunction and down regulation of transforming growth factor beta (TGF-β) receptors[6]
- Avoiding terminal differentiation of cells: In normal condition, certain fraction of proliferating cells goes to the terminal differentiation and leaves the cell cycle forever. By this process, the normal tissue checks the cell proliferation. However, the cancer cells take various strategies to avoid terminal differentiation. Cancer cells often overexpress c-Myc oncogene and produce increased c-Myc proteins.

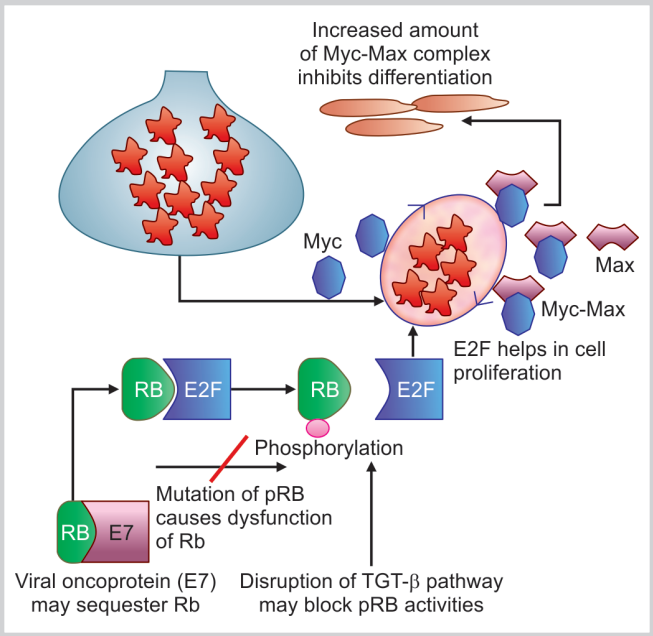


Fig. 5.10: Diagram shows how the cancer cells evade the influence of antigrowth signals. Retinoblastoma and p53 pathway of the cell-cycle inhibition is disrupted due to the mutation of p53 or Rb gene. Rb and p53 gene products may also be sequestered by viral oncoproteins. There may be mutation, dysfunction, and downregulation of transforming growth factor beta receptors causing disruption of inhibitory pathway of Rb protein. Excess Myc protein may bind with Max and Myc-Max complex may have inhibitory effect on cell differentiation

This c-Myc protein binds with Max and increased Myc-Max complex, impairs differentiation, and enhances cell proliferation.

Resisting Cell Death or Evading Apoptosis

Cancer cell has the ability to resist apoptosis. This capability is achieved by a variety of strategies (**Fig. 5.11**):

- In the vast majority of the malignant tumor, the cancer cell blocks the functional activity of p53 protein by mutation of p53 gene that normally acts as proapoptotic[7]
- Cancer cells may also resist apoptosis by enhancing the expression of the antiapoptotic signals such as B-cell lymphoma 2 (BCL2) or by downregulating proapoptotic signals (Bax)
- Death ligand Fas binds to the death receptor and causes apoptosis. Cancer cell deregulates this Fas death receptor pathway.[8]

Limitless Replicative Potential

The cancer cell acquires the limitless potential to replicate. The normal cells have limited number of cell division and proliferation. After a certain number of proliferative cycles, the

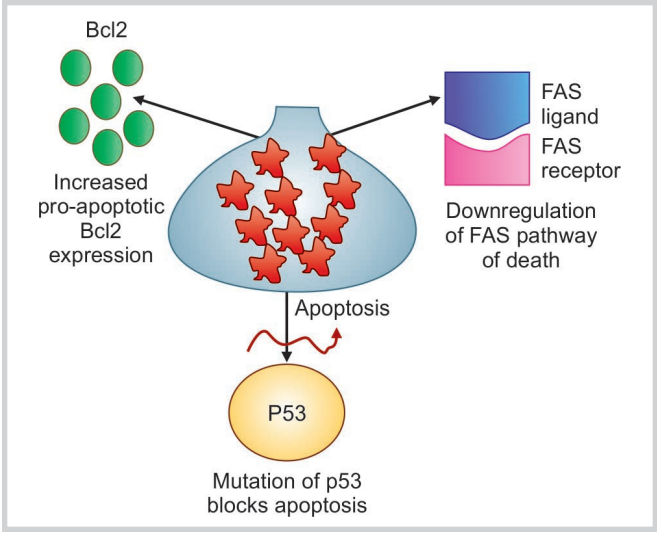


Fig. 5.11: Schematic diagram shows the mechanisms of blockage of apoptosis. This blockage of apoptosis may be done by downregulation of Fas receptor pathway, mutation of p53, and increased production of proapoptotic protein Bcl2

normal cells have to cross two distinct barriers of proliferation, senescence and crisis phase. Senescence is the irreversible nonproliferative but viable state. The certain fraction of cell undergoes to the crisis phase and dies. This replicative barrier or checkpoint is maintained by telomeres of the chromosome. Telomeres are the multiple repeats of hexanucleotide sequence that protect the ends of the chromosomal deoxyribonucleic acid (DNA). There is progressive loss of telomere at the end of each cell cycle. After the end of certain number of cell cycles, the telomere shortens significantly and triggers the cell to enter into crisis phase of death.[9] Unlike the normal cell, the tumor cells upregulate the expression of the telomerase enzyme and preserves the length of the telomere by adding the repetitive nucleotide sequence onto the end of the telomeric DNA. This prevents the cancer cells to enter into senescence phase and keep them in the continuous replicative phase (**Fig. 5.12**).[10]

Sustained Angiogenesis

The cancer cells try to maintain continuous supply of oxygen and nutrients by the sustained angiogenesis. They activate the angiogenic switch by increased expression of angiogenic factors, such as vascular endothelial growth factor and fibroblast growth factor (FGF) and downregulating the antiangiogenic factors, such as thrombospondin-1 and β-interferon.[11,12]

However, the neoangiogenesis in cancer is aberrant and is characterized by precocious capillaries, excessive branching of the blood vessels, microhemorrhage, and distorted vessels with erratic blood flow.[13]

Tissue Invasion and Metastasis

Invasion to the local tissue and distant metastasis are two unique characteristics of the cancer cell. Both local invasion and

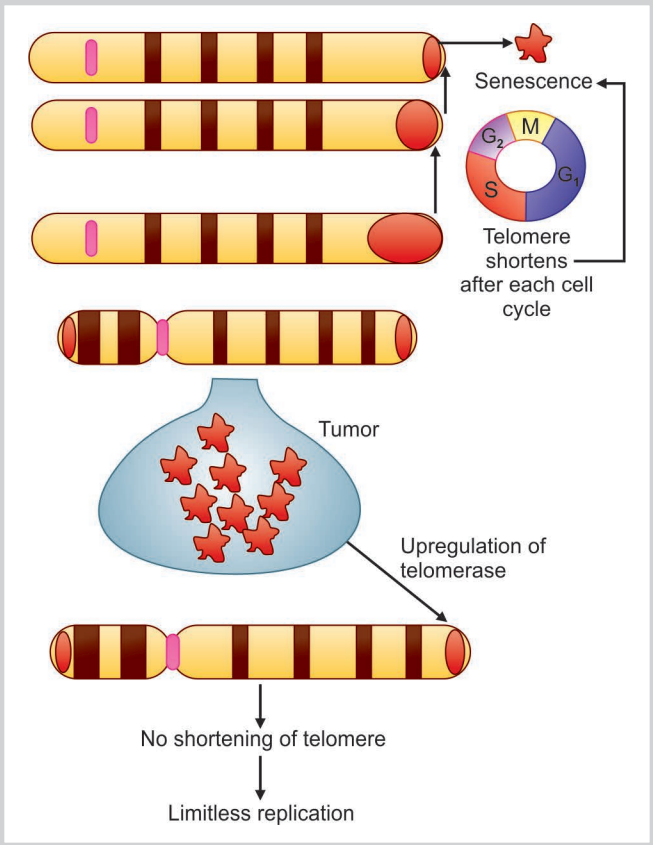


Fig. 5.12: Schematic diagram shows how the tumor cells gain limitless replicative potential. At the time of each cell cycle, the telomere portion of DNA shortens. The cell undergoes senescence when the telomere reaches to a critical length. Tumor cells produce telomerase enzyme that prevents the reduction of telomere length. Therefore, the cells do not enter in senescence phase and gain limitless replicative potential

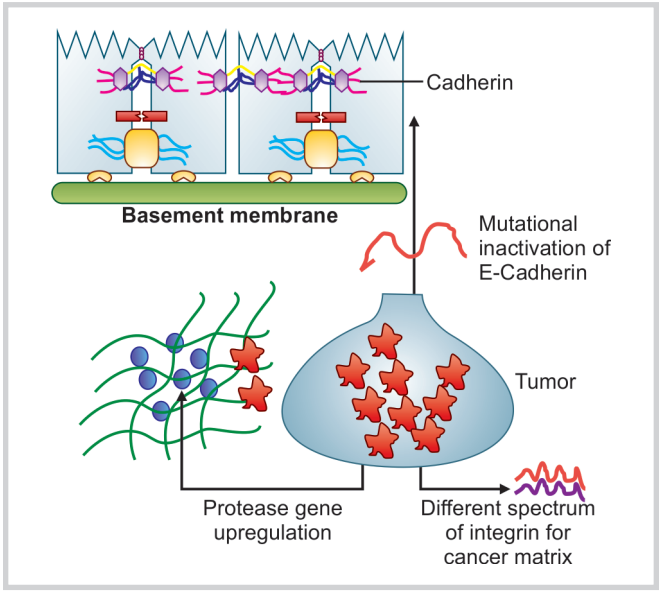


Fig. 5.13: The schematic diagram shows the mechanisms of invasion of cancer cells. E-cadherin maintains cell-to-cell adhesion. Mutation of E-cadherin in cancer cell causes disaggregation of the tumor cells. Simultaneously, increased amount of protease or downregulation of the protease inhibitors helps in tissue invasion by cancer cell. Cancer cells also produce different types of integrins that make cancer-specific tissue matrix

metastasis are well-related events. Metastasis of cancer cell is achieved by (**Fig. 5.13**):

- Downregulation or mutational inactivation of epithelial cadherin: Epithelial cadherin (E-cadherin) is a cell-to-cell adhesion molecule. It helps form sheets of epithelial cells by adhering two cells together. Epithelial-cadherin expression is a key molecule that prevents metastasis. In majority of cancer, the function of E-cadherin is lost and metastasis occurs[14]
- Changing the type of integrin expression: Cancer cell produces different spectrum of integrins (integrin α and β) that best fit with the tissue matrix of the metastatic site
- Liberation of protease: Proteases genes are upregulated and inhibitors of proteases are downregulated by cancer cell. The increased protease activity helps in tissue invasion and metastasis.[15]

Reprogramming Cellular Metabolism

Cancer cells reprogram their glucose metabolism and even in the presence of oxygen cancer cell gets energy from glucose by glycolysis.[16]

Genomic Instability and Mutation

Negrini S et al. considered that genomic instability is an emerging hallmark of cancer cell.[17] Chromosomal instability (ChIN) is one important form of genomic instability by which chromosome number and structure is changed in course of tumor development. Microsatellite instability (MSI) is another cause of genomic instability. In case of MSI, there is an expansion or contraction of the number of oligonucleotide repeats present in microsatellite sequences. Genomic instability causes increased spontaneous mutation rate in cancer cells and makes the cancer cell more aggressive.[18]

Tumor-promoting Inflammation

Inflammation contributes to the progression of tumor by providing bioactive molecules to tumor microenvironment, growth factors, proangiogenic factors, and extracellular matrix-modifying enzymes. Tumor-promoting inflammation is considered as one of the emerging hallmark of cancer.[19]

Evading Immune Destruction

Normal human immune system makes significant barrier in the development and progression of tumor. Cancer cell evades the immune mechanism of the body by secreting TGF-β or other immunosuppressive factors and also by recruiting immunosuppressive T regulatory cell.[20,21]

BOX 5.1 Cancer stem cell

- A malignant cell with the functional properties of stem cell
- Characteristics of cancer stem cell:
  - Poorly regulated self-renewal
  - Unlimited proliferative capacity (resistant to apoptosis)
  - Differentiates to produce tumor
  - Long life span
- Stem cell model of cancer: Only specific stem cells undergo mutation to produce tumor rather than differentiated cell
- Therapeutic significance: Cancer stem cells are slowly growing and difficult to eradicate

BOX 5.2 Multistep process of carcinogenesis

- Cumulative changes of genetic abnormalities in the tumor cell
- Two types of abnormalities:
  - Deoxyribonucleic acid sequence change
  - Epigenetic change causing gene silencing
- Each mutation or genetic changes provides additional advantages of the tumor cell
- Best studied in colonic carcinoma

CANCER STEM CELLS

Stem cells have certain unique characteristics: (1) self-renewal, (2) the potential to develop into multiple lineages, and (3) unlimited proliferative capacity.[22] Self-renewal is a crucial function of stem cell. By the process of self-renewal, the daughter cell derived from the stem cell retains their biological properties like the parent cell. Stem cells are rarely found in the adult tissue, and therefore it is difficult to study them. There is no morphologically identifiable characteristic of stem cell. A malignant cell with the functional properties of stem cell is called as "cancer stem cell". Cancer stem cells like normal stem cells have immense self-renewal capacity, long life span, and ability to resist apoptosis (**Box 5.1**). Like somatic stem cells they also can migrate to distant tissue. Somatic stem cells generate specific tissue in highly regulated way, whereas cancer stem cells are the source of tumor production in aberrant poorly regulated way. Cancer stem cells are well described in hematological malignancies, breast and central nervous system tumors.[23-25] Most probable origin of cancer stem cell is from mutation of normal progenitor cells.[26] These progenitor cells poses the replicative capacity, but they usually lack self-renewal ability. The unique characteristic of self-renewal capability is gained by acquired mutation of the progenitor cell. Significance of the cancer stem cell is related with the failures of cancer treatment, because of slow growing property of the cancer stem cells. Therefore, cancer therapy should be specifically targeted to cancer stem cell for its success.

MOLECULAR BASIS OF CANCER

Multistep Process of Carcinogenesis

The development of cancer is a multistep process, which consists of cumulative changes of genetic abnormalities in the tumor cell. The genetic abnormalities may be in two ways (**Box 5.2**):

1. Deoxyribonucleic acid sequence change
2. Epigenetic change causing gene silencing.

Kinetic analysis showed that the cancer cell undergoes at least six rate-limiting mutations before it becomes clinically evident.[27] Each mutation or genetic change provides additional advantages of the tumor cell. In addition, the loss of the normal control of the mutation may probably help in stable mutations in the genome of the affected cells and possibly neoplastic transformation happens when some "critical transformation-associated target genes" are damaged. This multistep process is well illustrated in colonic carcinoma which typically develops over decades and passes through adenoma to carcinoma sequences. During this transformation, there are at least 6–7 mutational changes occurring that take vital role in neoplastic transformation. Mutation of adenomatous polyposis coli (APC) gene is the rate-limiting step that initiates the tumorigenesis of normal colonic epithelial cells. This is followed by successive mutation of K-RAS, deleted in colorectal carcinoma and p53 genes (**Fig. 5.14**).[28]

The precise nature and the sequence of the genetic or epigenetic abnormalities in other malignancies are not known. It is possible that each tumor has its own sequence of genetic or epigenetic changes, and there may be immense complexities in the sequence of genetic changes.

CLONAL EVOLUTION OF CANCER

Few decades ago, Nowel PC first time proposed the clonal origin of cancer.[29] Today, it is of beyond doubt that cancer evolves as clonal proliferation and selection of group of cells that are originated from single precursor cell (**Fig. 5.15**). The evolution and progression of cancer is just like a complex Darwinian adaptive system.[30]

In earth, evolution of the habitat took place in the ecosystem whereas in tumorigenesis the evolution of the cancer cell happens in the microenvironment of the tissue. The evolving cancer cell tries to fit itself for space and nutrient. Possibly blind and purposeless multiple mutations of the DNA sequence occur in the evolving cancer cells. In course of time, the cancer clone emerges through the selective advantageous mutations that help in limitless proliferation, self-renewal, insensitive to antigrowth factors, evasion of apoptosis, angiogenesis, and metastasis. The exact kinetics of the clonal evolution of cancer largely depends on mutation rate (and also epigenetic changes) and clonal expansion. The rate of epigenetic changes is much more than mutational changes.[31]

ONCOGENES AND CANCER

Oncogenes are regulatory genes involved in numerous intracellular signaling pathways and promote autonomous cell

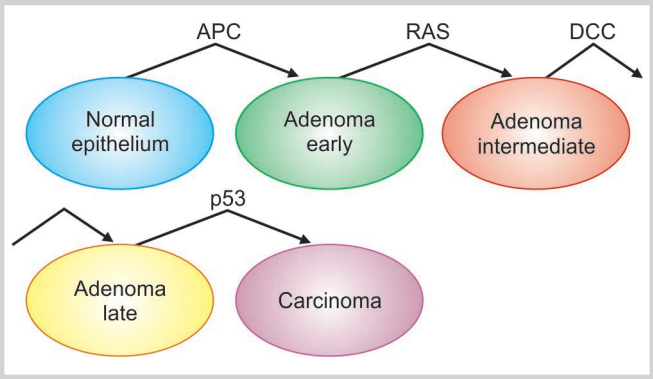


Fig. 5.14: The schematic diagram shows the process of multistep carcinogenesis in colonic adenoma to carcinoma. The various crucial genetic mutations happen in each step. Mutation of adenomatous polyposis coli gene is the rate limiting step of the carcinogenesis. After this mutation, the tumor subsequently undergoes various other genetic mutations

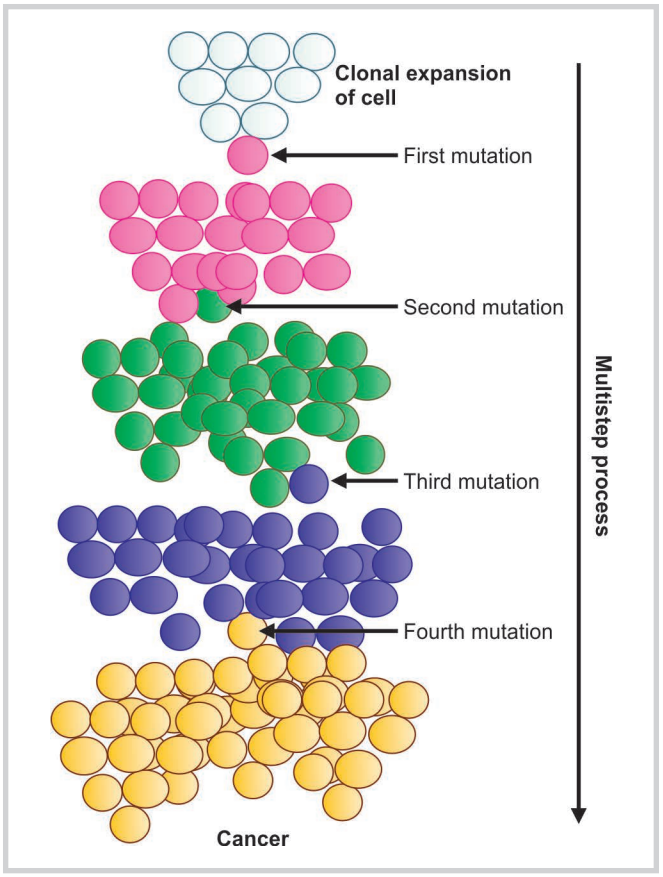


Fig. 5.15: The schematic diagram shows the clonal evolution of cancer. In each successive mutation, the cancer cells gain certain unique properties and become more and more aggressive

growth of cancer cells. The proto-oncogenes are normally present in the cell. They are the normal cellular counterpart of oncogenes in healthy cell. Proto-oncogene encodes various proteins that help in normal physiological cell growth and proliferation. Products of proto-oncogene are under direct regulatory control of the cell whereas the oncoproteins encoded by oncogenes are autonomous. Oncogene acts in dominant way that means if one of the two alleles of the genes is altered then the oncogene will show its biological action. A wide variety of oncogenes have been described so far (**Table 5.3**). These oncogenes are also localized in different parts of a cell, such as cell membrane, cytoplasm, and nucleus to do wide range of functions.

Activation of Oncogenes

Oncogenes are activated by various ways (**Fig. 5.16**):

- Chromosomal translocation
- Mutation
- Gene amplification.

Chromosomal Translocation

Fusion gene: Chromosomal translocation may cause formation of chimeric fusion gene and thereby produces chimeric protein with excessive tyrosine kinase activity or involving transcription factors.

TABLE 5.3: Oncogenes: position, function, and related with diseases

Oncogene	*Chromosome*	*Position*	*Mode of activation*	*Function*	*Disease*
H-RAS	11	Membrane	Point mutation	Signal transduction	Colon, lung, pancreas
K-RAS	12	Membrane	Point mutation	Signal transduction	AML, thyroid carcinoma
ERB-B2	7	Membrane	Amplification	Growth factor receptors	Breast and ovarian cancer
RET	10	Membrane	Point mutation	Growth factor receptors	Multiple endocrine neoplasia 2A, 2B
SIS	20	Membrane	Overexpression	Growth factor	Osteosarcoma
C-MYC	8	Nucleus	Translocation	Transcriptional activator	Burkitt's lymphoma
N-MYC	8	Nucleus	Amplification	Transcriptional activator	Neuroblastoma

Abbreviation: AML, acute myeloid leukemia

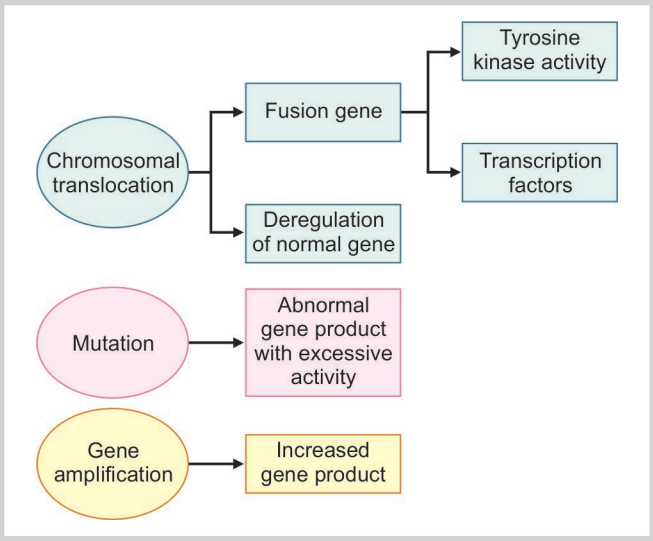


Fig. 5.16: The schematic diagram shows the various mechanisms of activation of oncogene and their effects. The three major ways of oncogene activation are chromosomal translocations, mutation and gene amplifications

Tyrosine kinase activity: BCR-ABL1 fusion gene in Philadelphia chromosome is the classic example of chimeric fusion gene causing excessive tyrosine kinase activity.

ALK-NPM1 fusion oncogene is generated by t(2;5)(p23;q35) in case of anaplastic large cell lymphoma.

Transcription factors: Chromosomal translocation can cause fusion protein with aberrant transcriptional activities. In Ewing's sarcoma, there is t(11:22) (q24:q12) translocation that produces ERG-EWSR1 fusion gene with excessive transcription factors activity.

Deregulated Expression of Structurally Normal Genes

Chromosomal translocation may often bring the proto-oncogene into a new position, which is under the influence of gene promoter or enhancer sequence of the other normal tissue regulatory element. Therefore, there may be excessive expression of the proto-oncogene. In case of Burkitt's lymphoma, c-Myc oncogene is transferred near the heavy chain gene onto chromosome 14 and is upregulated by the neighboring promoter.

Gene Amplification

Gene amplification is seen in many advanced cancers and multiple copies of the gene are noted in the cancer cells. Several hundred kilobases of DNA segment is usually amplified that contains many genes. Usually, oncogene families of MYC, epithelial growth factor receptors (EGFR), RAS and cyclin D1 are amplified. Amplification of ERB-B2 is usually seen in breast cancer and its overexpression correlates with poor prognosis.[32] MYC is amplified in small cell lung cancer, cervical cancer, breast and esophageal cancer.[33]

Mutation

Mutation in the coding sequence of the oncogene may cause abnormal gene product with increased activity, such as point mutation in RAS gene encodes a protein that remains continuously in active state and incessantly stimulates the mitogen-activated protein (MAP) kinase pathway to transmit growth promoting signals to the nucleus.[34] Point mutations of BRAF gene encodes a protein with change of valine residue at position 599 to glutamic acid in the kinase domain. This aberrant BRAF protein continuously stimulates MAP kinase cascades resulting incessant cell proliferation.[35]

FUNCTIONAL PROPERTIES OF ONCOGENE

Oncogenes perform their function by the production of oncoproteins that can be classified as (1) growth factors, (2) growth factor receptors (3) signal transducers (4) transcription factors (5) chromatin remodelers and (6) apoptosis regulators (**Box 5.3**).

Growth Factors

Cancer cells often overexpresses various growth factor genes that produce growth factors. SIS oncogene is overexpressed in osteosarcoma and low grade astrocytoma[36] resulting in excessive production of platelet-derived growth factor beta that causes unrestricted cell proliferation. HST-1 and INT-2 genes are overexpressed in stomach, breast and bladder carcinoma and produce the oncoproteins and FGF.

Growth Factor Receptors

Growth factor receptor has three parts: external ligand-binding domain, membranous domain and cytoplasmic domain.

BOX 5.3 Functional activity of oncogene

- Growth factors: Overexpression of various growth factor genes, e.g. SIS oncogene in osteosarcoma, HST-1 and INT-2 genes in stomach, breast, and bladder carcinoma
- Growth factor receptors:
  - Oncogenes produce abnormal growth factor receptors: activated without any binding with its ligand
  - Amplification of receptors,: e.g. ERB-B2 gene is amplified in breast and ovarian cancer with excess EGF receptors
- Signal transducers: Cytoplasmic signal transducing proteins with persistent activity, e.g. point mutation of RAS oncogene generates persistent activated-RAS protein
- Transcription factors: Chimeric fusion gene with aberrant activity of transcription factor
- Chromatin remodelers: Oncogene plays critical role in chromatin remodeling by modification of histones of the nucleosomes

Abbreviation: EGF, epithelial growth factor

Normally, when the ligand binds with external ligand binding domain of the growth factor receptors there is transient activation of tyrosine kinase enzyme by dimerization followed by phosphorylation of various substrates. Oncogenes may produce the growth factor receptors that are persistently activated without any binding with its ligand (**Fig. 5.17**). In multiple endocrine neoplasia 2A and 2B, there is point mutation of RET gene resulting encoding of the oncoproteins that has persistent activity of cytoplasmic domain of the tyrosine kinase receptors.

ERB-B2 gene is amplified in breast and ovarian cancer causing excessive production of EGFR, which is responsible for cell proliferation. In large number of squamous cell carcinomas, ERB-B1 oncogene is overexpressed resulting in excessive production of EGFR.

Signal Transducers

Oncogenes may produce oncoproteins that mimic cytoplasmic signal transducing proteins with persistent activity. RAS, the signal transducing molecule, is present in the inner surface of the cell membrane as guanosine-diphosphate (GDP) bound inactive form. When a normal cell is stimulated through a growth factor receptor, the inactive GDP bound RAS becomes active guanosine triphosphate (GTP) bound RAS. This is done by transforming GDP to GTP. After the stimulation, there is rapid hydrolysis of GTP to GDP and active RAS again reverts to inactive RAS. The point mutation of RAS oncogene generates persistently activated RAS protein as it is resistant to hydrolyze GTP to GDP. This is responsible for persistent stimulation of the receptor pathway without any external factors (**Fig. 5.17**).

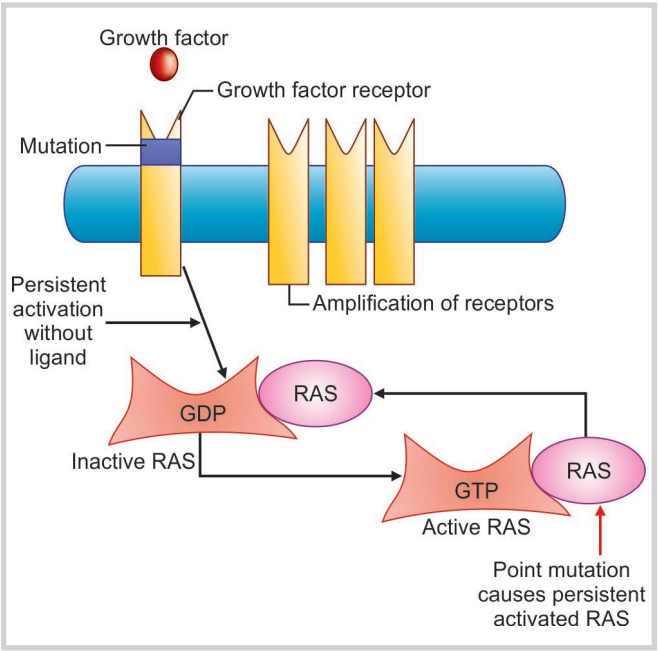


Fig. 5.17: The schematic diagram shows the mechanism of persistent signal transduction in cancer cell. Normally, after binding the growth factor and its receptor, the cytoplasmic domain of the receptor converts inactive guanosine diphosphate bound RAS to active guanosine triphosphate bound RAS. This active RAS becomes inactive again when guanosine triphosphate is hydrolyzed to GDP. In case of cancer, there may be mutation of RAS and the active form remains persistently active. There may be also mutation of growth factor receptors and the receptor may be activated without any growth factor

Oncogenes encode oncoproteins that simulate members of signal transduction pathway of both nonreceptor protein kinases and guanosine-triphosphate-binding proteins. The nonreceptor protein kinases are (1) Tyrosine kinases and (2) serine and threonine kinases.[37]

Transcription Factor

As mentioned before, chromosomal translocation often produces chimeric fusion gene with aberrant activity of transcription factor. In Ewing's sarcoma, there is t(11:22)(q24:q12) that produces ERG-EWSR1 fusion gene with excessive transcription factors activity. In carcinoma of lung and neuroblastoma, there is N-MYC amplification causing excessive production of transcription factor resulting in cell proliferation.

Chromatin Remodelers

When a portion of chromatin opens up, the transcription factors get a chance to interact with promoter regions of DNA. Therefore, chromatin remodeling is important in the control of gene expression. Oncogene plays critical role in chromatin remodeling. There are two kinds of enzymes that regulate chromatin remodeling: (1) adenosine triphosphate-dependent enzymes and (2) histone modifiers.[38] In case of acute lymphatic leukemia and acute myeloid leukemia, chromosomal translocation of 11q23 produces chimeric fusion oncogene ALL1 (mixed lineage leukemia) . The fusion protein of ALL1 is involved with chromatin remodeling by modification of histones of the nucleosomes.[39]

MICRORIBONUCLEIC ACID AND CANCER

Microribonucleic acids (miRNAs) are small, evolutionarily conserved RNA molecules that negatively regulate gene expression at the post-transcriptional level (**Box 5.4**).

Biogenesis of Microribonucleic Acid

Microribonucleic acid genes are located in the DNA as single gene or gene clusters. Microribonucleic acid genes may be located in the intronic coding, intronic noncoding region and exonic noncoding region. The miRNA genes are transcribed by RNA polymerase II and primary miRNAs (pri-miRNA) are formed. The pri-miRNA is like hairpin loop like structure and is cleaved by RNase III endonuclease Drosha to precursor miRNA (pre-miRNA). This pre-miRNA is transported from nucleus to cytoplasm. In the cytoplasm, pre-miRNA is converted to mature miRNA by the enzyme RNase III endonuclease Dicer. Mature miRNA is a single-strand RNA consisting of 16–23 nucleotides (**Fig. 5.18**).

BOX 5.4 Microribonucleic acid

- Definition: Microribonucleic acids (miRNA) are short, evolutionarily conserved, single-stranded RNA molecules
- Main function: Anneals to the complementary strand of messenger ribonucleic acid and causes translational repression and/or degradation
- Role:
  - Cellular differentiation
  - Cell proliferation
  - Apoptosis
  - Stem cell maintenance

Microribonucleic acid in cancer:

- Abnormally expressed in large number of cancer
- Helps overcome the cellular senescence and apoptosis
- Loss of miRNA causes unopposed expression of BCl-2

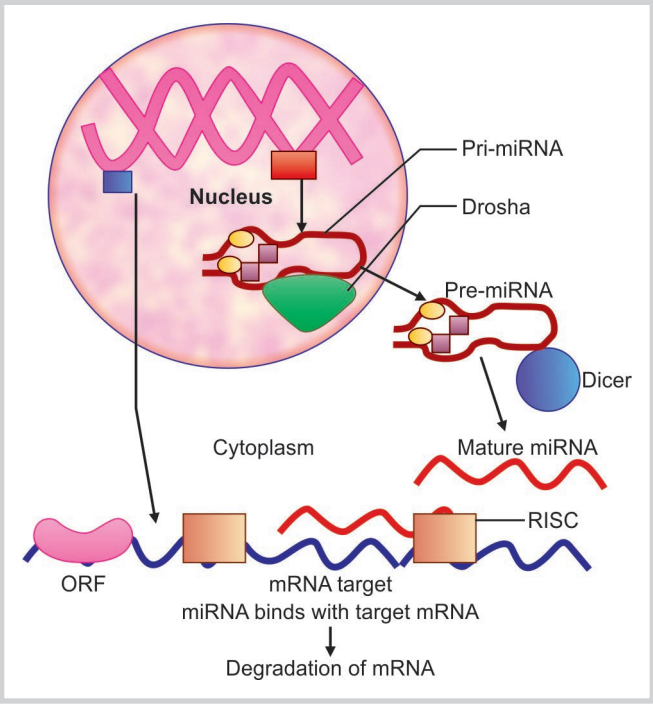


Fig. 5.18: The schematic diagram shows microRNA formation and its function. At first primary microRNA (pri-microRNA) is formed followed by pre-microRNA and mature microRNA. This mature miRNA combines with mRNA at specific sites and blocks its action

Function

Microribonucleic acid molecule anneals to the complementary strand of messenger RNA leading to translational repression and/or degradation.[40] It has been suggested that genes of miRNAs play a significant role in cellular transformation and carcinogenesis acting either as oncogenes or tumor suppressors.[41]

In recent years, the role of miRNA has been described in cellular differentiation, cell proliferation, apoptosis, and stem cell maintenance.[42,43]

Gene mapping has shown the presence of large number of miRNA genes in the chromosomal regions that are frequently affected in cancer cells.[44] Microribonucleic acid expression profiles have shown its association with tumor diagnosis and progression.[45] Many miRNAs are abnormally expressed in large number of cancer, such as acute lymphatic leukemia, glioblastoma, Burkitt's lymphoma, breast, colon, lung and liver cancer.[45,46] Possibly, miRNA expression interferes the function of critical gene expression of tumor cells in relation to cell proliferation and differentiation.

Microribonucleic Acid with Oncogene Activity

miR-372 and miR-373 are considered as oncogene in testicular germ cell tumor.[47] These miRNAs help to overcome the cellular senescence and apoptosis induced by p53 gene by directly neutralizing the p53-mediated cyclin-dependent kinase 2 (CDK2) inhibitions and subsequent cell cycle arrest. Microribonucleic acid-155 also behaves as oncogene and overexpression of miRNA155 is noted in diffuse large B-cell lymphoma, chronic lymphocytic leukemia, Burkitt's lymphoma, and nonsmall cell lung carcinomas.[46,48-50]

Microribonucleic Acid with Tumor-suppressor Activity

Microribonucleic acid gene is deleted or downregulated in many cancers such as in B-cell chronic lymphocytic leukemia (B-CLL), mantle cell lymphomas, prostate cancer, and multiple myeloma.[51] Deletion of 13q14, is observed in B-CLL with loss of miR-15a and -16-1 genes. Possibly miR-15a and miRNA16-1 target the expression of antiapoptotic gene BCl2.[52] The loss of miRNA-15 and 16-1 causes unopposed expression of BCl2.

GENOMIC INSTABILITY

Genomic instability is considered as one of the important causes of tumorigenesis.[2] The genomic instability may occur by various ways:

- Chromosomal instability
- Microsatellite instability
- CpG island methylator phenotype
- In addition, potentially other, presently not yet identified mechanism may be present.

Chromosomal Instability

It is one of the important ways to develop genomic instability. Chromosomal instability is defined as a persistently high rate of loss and gain of whole chromosomes or part of the chromosome.[53] The result of ChIN is the loss of heterozygosity (LOH) or imbalance of the number of chromosome (aneuploidy).

In case of ChIN, there is defect in proper segregation of chromosome in the mitotic phase. This may be due to (1) cohesion defects, (2) spindle assembly checkpoint (SAC) defects (3) supernumerary centrosomes, (4) defects in Kinetochore-

microtubule attachment, and (5) defects in cell cycle regulation (**Box 5.5**).

Cohesion Defects

Certain proteins are responsible for cohesion of sister chromatids. Acute depletion of such proteins elevates the number of tetraploid cells.[54] Similarly, overexpression of separase also increases the incidence of tetraploid cells.[55]

Spindle Assembly Checkpoint Defects

This checkpoint regulates the proper alignment of the chromosome in the equatorial plate in anaphase and successful delivery of a correct chromosome set to each daughter cell. Defects in the SAC may cause aberration in anaphase leading to chromosome mis-segregation and aneuploidy. The proteins encoded by hBUB1 and MAD2 are two important regulatory molecules of the SAC.[56,57] Mutations of SAC genes have been identified in breast, colon, and gastric cancer cell lines.[58] However, the role of SAC in the occurrence of ChIN in clinical cancer is still a matter of debate.

Supernumerary Centrosomes

The centrosome amplification is seen due to overexpression of Aurora A kinase.[59] Extracentrosomes may produce multiple spindle formation leading to tetraploidy. The tetraploid cells are prone to have ChIN.

Defects in Kinetochore-Microtubule Attachment

Microtubule attachment to kinetochore is reversible. The repeated association and dissociation of microtubules from the kinetochore generates a dynamic kinetochore-microtubule association. This is helpful in error correction of microtubule attachment. Excessive stabilization of microtubule-kinetochore attachment causes defect in the kinetochore attachment leading to aneuploidy. Aurora B kinase is central to this control system. However, perturbation of other proteins such as MCAK, CNEP-E and APC, etc. are also involved in defective microtubule-kinetochore attachment.[60-62]

Defects in Cell Cycle Regulation

A large numbers of cell cycle regulator genes are also involved in ChIN such as BRCA1, BRCA2, Rb, p53, Mdm2, etc.[63] These cell cycle regulatory genes possibly promote ChIN by chromosome mis-segregation, centrosome duplication during S phase, or allowing defective chromosome to undergo mitosis.

Microsatellite Instability

Microsatellites are stable inherent, short repetitive sequence of 1–6 base pair of DNA (**Box 5.6**). The repetitive unit may be mono, di, tri, tetra, penta or hexa nucleotides. This is unique for each individual. Microsatellites are distributed not exactly randomly. They have the tendency to be remained clusters. Microsatellite instability means alteration of the pattern of polymorphic short tandem repeat segments of DNA. Normally, during replication of DNA, there may be mutation of microsatellite. However, the rate of microsatellites mutation is very low. In case of carcinoma, the initial mutation of microsatellite remains, and this rate of mutation increases due to clonal proliferation of cells. Large number of mutations in microsatellite sequences is observed in different carcinomas particularly colonic carcinoma.[64] This MSI is also associated with the mutation of several DNA mismatch repair genes, such as hMSH2, hMSH6, hMSH3, hMLH1, hPMS1, and hPMS2[64] (**Fig. 5.19**). Therefore, the presence of MSI reflects the

BOX 5.5 Chromosomal instability

Definition: Persistently high rate of loss and gain of whole chromosomes or part of the chromosome
Result:
- Loss of heterozygosity or
- Imbalance of the number of chromosome (aneuploidy)

Mechanisms:
- Cohesion defects: Depletion of proteins responsible for cohesion of sister chromatids
- Spindle assembly checkpoint defects: Regulates the proper alignment of the chromosome in the equatorial plate in anaphase
- Supernumerary centrosomes: Centrosome amplification due to overexpression of Aurora A kinase
- Defects in Kinetochore-microtubule attachment: Excessive stabilization of microtubule-kinetochore attachment
- Defects in cell-cycle regulation: Mutation of cell-cycle regulator genes, such as BRCA1, BRCA2, Rb, p53, Mdm2, etc.

BOX 5.6 Microsatellite instability

- Microsatellites:
  - Stabile, inherent
  - Short repetitive sequence of DNA
  - Unique to each individual
- Microsatellite instability: Alteration of the pattern of polymorphic short tandem repeat segments of DNA
- Genes commonly mutated in mismatch repair genes: hMSH2, hMSH6, hMSH3, hMLH1, hPMS1 and hPMS2
- Microsatellite instability is responsible for multiple mutations of cancer-related genes
- A marker of genomic instability
- Microsatellite instability: Noted in colonic, gastric, esophageal and endometrial cancer
- Microsatellite instability is measured by polymerase chain reaction

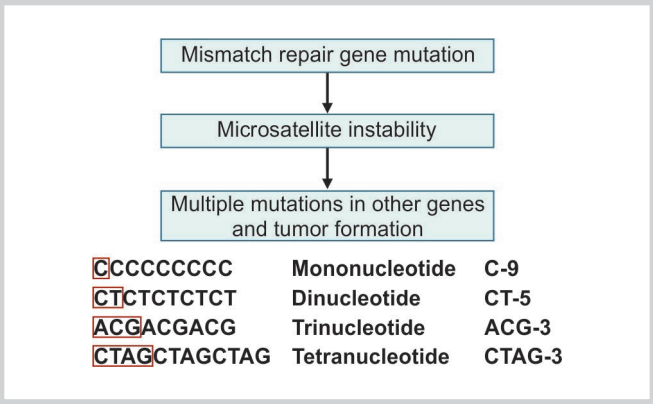


Fig. 5.19: The schematic diagram shows that mismatch repair genes are responsible for microsatellite instability and subsequently tumor formation. Microsatellite may be formed due to repetition of one, two, three, or more nucleotides

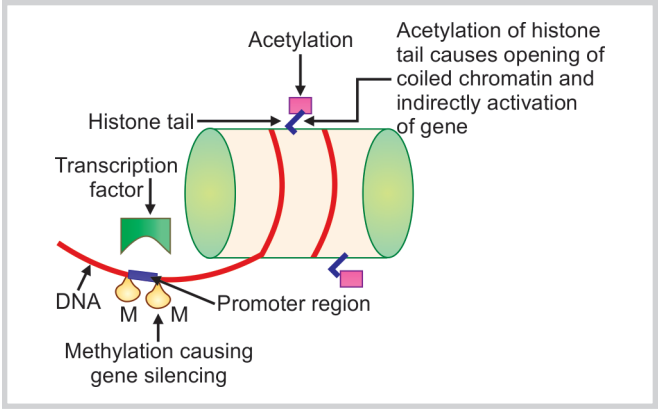


Fig. 5.20: The schematic diagram shows various mechanism of DNA silencing. Methylation of cytosine at the CpG islands of DNA promoter sites causes silencing of the respective gene. In addition, acetylation of histone tail may cause gene activation, whereas deactylation may be responsible for gene silencing

inactivation of mismatch DNA repair system. This genomic instability further accelerates the mutations of the other cancer promoting genes and provides various advantageous conditions to the tumor. The length of the microsatellites varies in the tumor tissues, and this is possibly due to multiple insertion and deletion of DNA.[65] Microsatellite instability has been first described in colorectal cancer.[66] However, MSI is also noted in other human solid tumors, such as esophageal, gastric and endometrial carcinomas. Microsatellite instability is considered as a marker of genomic instability. Microsatellite instability can be demonstrated by PCR amplification of genetic loci of microsatellite. According to Bethesda guidelines, if more than 30% of the microsatellite marker panel is mutated then it is labeled as MSI-high.[67]

CpG Island Methylator Phenotype

So far, we have discussed various genetic changes in cancer. However, now we know that many epigenetic changes are also responsible for carcinogenesis in the form of gene silencing by DNA methylation and histone modification.[68] Methylation of cytosine at the CpG islands of DNA occurs by addition of a methyl group to the carbon-5 position of cytosine with the help of DNA methyltransferase enzymes. Specific DNA methylation in the promoter region of DNA causes silencing of the respective gene (**Fig. 5.20**). In a malignant cell, there may be demethylation of normally hypermethylated region and conversely, the many unmethylated CpG islands containing genes may be hypermethylated. Therefore the genes responsible for cell-cycle control, DNA repair or apoptosis may be inactivated by DNA hypermethylation.[69,70] There is no doubt that DNA hypermethylation is a significant epigenetic change in carcinogenesis. However, the initial triggering factor of hypermethylation is unknown. Possibly, this occurs as an early event in carcinogenesis affecting the tumor-suppressor genes.

TUMOR-SUPPRESSOR GENES

Tumor-suppressor genes are normally present in the cell and regulate the physiological cell division and growth. These genes function as a negative regulator of oncogenes and cell-cycle checkpoints and protect the body against tumor development. Tumor-suppressor gene is recessive, and therefore, both alleles must be mutated before biological effect is noted.

Knudson's Two-hit Hypothesis

In relation to tumor-suppressor gene, it is important to mention two-hit hypothesis theory proposed by Knudson AG.[71] He suggested that in children with early onset retinoblastoma, there is an inherited mutation in retinoblastoma gene which is present in all the somatic cells and a second or additional somatic mutation (second hit) in other allele may lead to retinoblastoma. In case of late onset retinoblastoma (sporadic cases) of the children, two somatic mutations occur in the single retinal cell and tumor develops (**Fig. 5.21**). This important hypothesis explains the presence and nature of the tumor suppressor genes. **Table 5.4** shows the location and functions of some essential tumor suppressor genes.

p53—Policeman of the Genome

This is the most widely studied tumor-suppressor gene so far. This gene is located in chromosome 17p13.1 (**Box 5.7**). The other family members of p53 tumor-suppressor include p63 and p73.[72] These proteins are functionally related with p53 and has clear role in normal development. Wild type p53 takes important role in cell-cycle control and apoptosis and is capable of suppression of tumor growth. Loss of heterozygosity of the wild type p53 may cause cancer. It is the most commonly mutated gene and more than 50% of the human cancer show p53 mutation. Mutant form of p53 has a much longer half-life than wild type. However, mutant p53 protein is devoid of any functional activity.

TABLE 5.4: Tumor-suppressor genes

Gene	*Chromosome*	*Location of encoded protein within cell*	*Protein function*	*Familial cancer association*	*Tumor associated with somatic mutation*
p53	17	Nucleus	Cell-cycle arrest and apoptosis	Li-Fraumeni syndrome	Many human cancers (over 50%)
Rb	13	Nucleus	Cell-cycle control	Retinoblastoma	Retinoblastoma, osteosarcoma, breast, lung and bladder carcinoma
APC	5	Cytoplasm	Binds and degrades β catenin, inhibition of signal transduction	Familial adenomatous polyposis	Colorectal cancer
WT1	11	Nucleus	Transcriptional regulation	Wilms' tumor	Wilms' tumor
PTEN	10	Cytoplasm	Cell cycle arrest and apoptosis	Cowden syndrome	Endometrial carcinoma, thyroid and prostate cancer
BRCA1, BRCA2	17/13	Nucleus	DNA damage repair	Familial breast and ovarian cancers	Not known
p16(INK4A)	9	Nucleus	CDK inhibitor	Melanoma	Breast, esophagus and pancreatic cancer

Abbreviations: Rb, retinoblastoma; APC, adenomatous polyposis coli; CDK, cyclin-dependent kinase; PTEN, phosphatase and tensin homolog; DNA, deoxyribonucleic acid

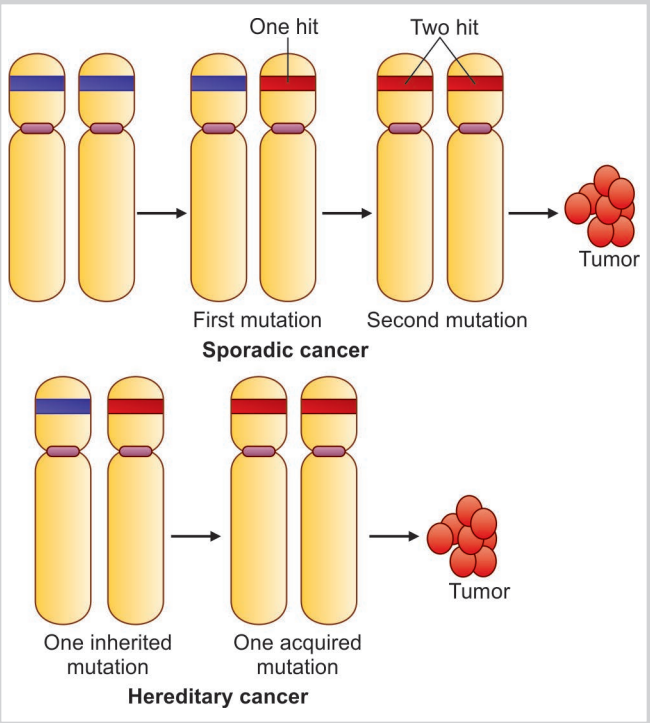


Fig. 5.21: The schematic diagram shows Knudson's two hit hypothesis of carcinogenesis. In case of sporadic cancer, two mutations occur in the somatic cells. The tumor develops when both the alleles undergo mutation. In hereditary cancer, there is an inherited mutation in one of the genes followed by another mutation of the allele

Mechanism of Loss of Function of p53

The function of p53 may be lost in various ways:

- Mutation: Usually missense point mutation occurs in the central sequence-specific DNA binding domain of p53. This mutation changes the conformation of the sequence-specific DNA binding domain of p53 and the transcriptional activity of p53 protein is lost. The mutant p53 protein is more stable with longer half-life than wild-type p53, and is present at very high levels in the tumor cell. In addition, the mutant form of p53 has oncogenic potential and can act as dominant negative inhibitor towards wild type p53.[73]
- Loss of heterozygosis: Loss of heterozygosity of wild type p53 has also been noted in many human cancers leading to the loss of function of p53.
- Deranged function due to inactivation by binding with other proteins: Various virally encoded proteins, such as SV40 large T antigen, the HPV E6 ORF, hepatitis B X antigen, and adenovirus E1a bind with wild type p53 protein and may inactivate its function.[7d]
- Mutations of regulators of p53: Mutation of the regulators or stabilizers of p53 protein may cause loss of p53 function. MDM2 protein activates and stabilizes p53 protein. Therefore, any defect in this pathway due to mutation of MDM2 may derange the function of p53.[75]

Functions of p53 and Carcinogenesis

p53 interacts with various target genes or proteins to perform its function (**Fig. 5.22**). It binds with the DNA sequence of the

BOX 5.7 **p53, the tumor-suppressor gene**

- Location: Chromosome 17p13.1.
- Wild type p53 takes important role in cell cycle control and apoptosis.
- Mutant p53 protein is devoid of any functional activity
- Mutant form of p53 has a much longer half-life than wild type
- The most commonly mutated gene in cancer

Mechanism of loss of function:

- Mutation
- Loss of heterozygosity
- Deranged function due to inactivation by binding with other proteins
- Mutations of regulators of p53

Functions of p53:

- Cell cycle arrest: Activated p53 protein produces p21 that inhibits CDK and prevents phosphorylation of pRb
- Repair of DNA damage: p53 activates GADD45 gene that encodes a protein related with DNA repair
- Apoptosis: Excess BAX protein and cell surface death receptors, such as DR5 and FAS are produced

Abbreviation: CDK, cyclin-dependent kinase; BAX, BCL2-associated X

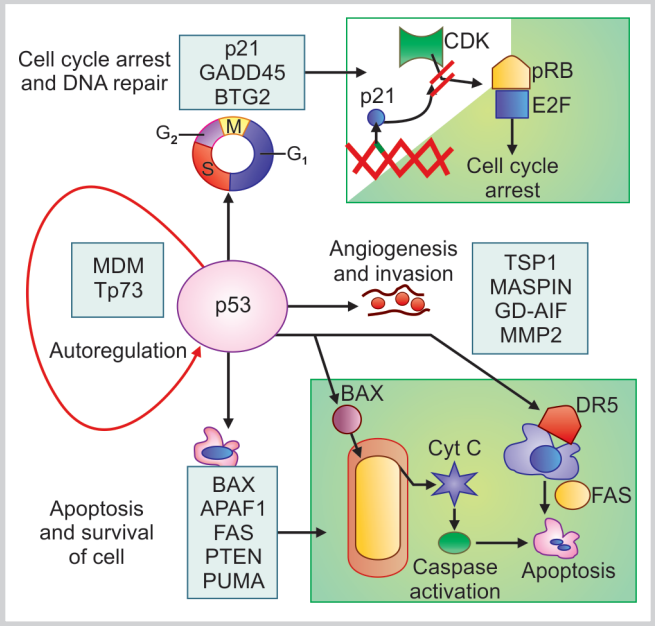


Fig. 5.22: The schematic diagram shows various target genes of p53. p53 is normally involved in cell-cycle control, apoptosis and DNA repair

regulator of the target genes and either activates or represses the genes. p53 also acts as nontranscriptional activator of apoptosis, signal transduction, and replication factor. It also interacts directly with many cellular proteins and several viral proteins.

Cell-Cycle Arrest

The main function of p53 is cell-cycle arrest, DNA damage repair and apoptosis. Initially p53 is activated by cellular stresses, such as DNA damage, telomere erosion, hypoxia, metabolic changes, cytokines, or activated oncogenes. The activated p53 protein binds with the specific DNA sequence of several target genes and stimulates their transcription. Predominantly p21, a CDK inhibitor and GADD45 are produced. Increased level of p21 prevents CDK to phosphorylate Rb protein leading to cell-cycle arrest in G1 phase.[76] In case of tumor, after DNA damage, the mutant p53 fails to block the cell cycle by p21 transcription. Therefore, the tumor cells proliferate with damaged DNA leading to genetic instability and multiple mutations.

Repair of Deoxyribonucleic Acid Damage

In case of DNA damage, p53 activates GADD45 gene that encodes a protein related with DNA repair. If the damaged DNA is not repaired successfully, then p53 stimulates the apoptosis-induced genes and induces apoptotic cell death.

Apoptosis

p53 induces apoptosis if the cell fails to repair its damaged DNA or fails to adapt up with stress. It induces various genes responsible for mitochondrial pathway of apoptosis. Induction of BAX gene by p53 protein causes excess BAX protein in mitochondrial membrane leading to increased mitochondrial permeability. This causes the release of cytochrome C from the mitochondria to cytoplasm and activation of caspase cascades that initiates apoptosis. p53 may also activate the other transcriptional targets related with cell surface death receptors such as DR5 and FAS. These receptors when bind with the respective ligands, activates the caspase pathway to initiate apoptosis.[77,78] Mutant p53 not only fails to initiate apoptosis and to promote tumorogenesis, but also enhances tumor cell resistance to death.[79]

Retinoblastoma Gene

It is the first tumor suppressor gene identified. Retinoblastoma gene is a recessive gene and both the gene should be inactivated before derangement of their function.

Inactivation of Retinoblastoma Gene

The gene is located in 13q14 location. The gene may be inactivated by mutation or deletion. Retinoblastoma gene produces Rb protein and this can be inactivated by binding with the viral products.

Functions of Rb protein (**Box 5.8**):[81]

- Cell-cycle checkpoint in G1 to S phase
- Cellular differentiation
- Cell survival
- Cellular senescence
- Genomic instability.

BOX 5.8 Retinoblastoma protein

- Retinoblastoma is a recessive gene located in 13q14 chromosome
- Inactivated by mutation or deletion
- Functions:
  - Cell cycle control:
    - pRb protein in its hypophosphorylated state binds with E2F transcription factor
    - Phosphorylated Rb releases E2F that helps in cell cycle progression
  - Chromatin remodeling: pRb protein binds with histone deacetylases, histone methyltransferases, histone demethylases, DNA methyltransferases, and other histone remodeling complexes that influence chromatin remodeling
  - Genomic instability: Functional inactivation of pRb may lead to aneuploidy, subchromosomal loss, endoreduplication, and consistently high rate of chromosomal mis-segregation
  - Deoxyribonucleic acid damage: pRB protein controls the expression of various DNA damage repair factors

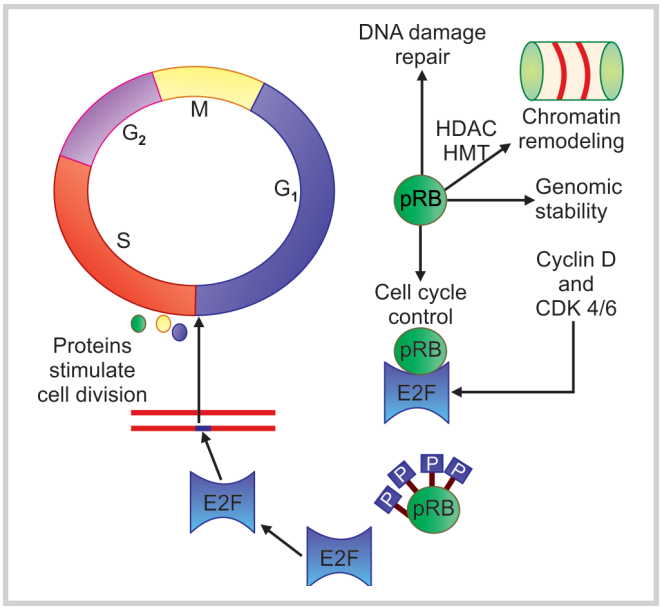


Fig. 5.23: The schematic diagram shows the various functions of pRB. pRB is related with cell-cycle control, genomic stability, DNA repair and chromatin remodeling

Cell-Cycle Control

pRb is an adaptor protein and can interact with large variety of cellular and viral proteins. pRb protein in its hypophosphorylated state binds with the sequence-specific DNA binding E2F transcription factor which is the product of E2F transcription gene.[81] Cyclin-cyclin dependent kinase complex phosphorylates pRB and releases E2F from the pRB-E2F complex. E2F is involved in the transcription of many genes that are essential for cell-cycle progression (**Fig. 5.23**). Thus, normally by blocking the action of E2F, pRb controls the cell-cycle entry of the cell. Deletion or mutational inactivation of pRb thereby may cause uncontrolled cell proliferation and cancer. The function of Rb gene and its products are blocked in large number of cancers.

Chromatin Remodeling

Transcription factors are only able to act on particular promoter sites and express specific gene when a portion of chromatin coil is opened up.

pRb protein binds with a host of factors that influence chromatin remodeling such as histone deacetylases, histone methyltransferases, histone demethylases, DNA methyltransferases and other histone remodeling complexes.[81] Chromatin remodeling plays essential role in gene expression.

Genomic Instability

The inactivation of pRB results in mitotic defects due to supernumerary centrosomes and centromeric defects. Various studies have demonstrated that the functional inactivation of pRb may lead to several types of genomic alterations, such as aneuploidy, subchromosomal loss, endoreduplication and consistently high rate of chromosomal mis-segregation.[82,83]

Deoxyribonucleic Acid Damage

pRB protein controls the expression of various DNA damage repair factors and these factors also may affect DNA repair processes indirectly.[85] Inactivation of pRB fails to check the damaged DNA to enter into the cell cycle and, therefore, renders cells more prone to DNA damage.

PRENEOPLASTIC LESIONS

Certain lesions of the epithelial origin are often restricted to the lining epithelium of the tissue and show well-defined association with cancer. These lesions have been labeled as precancerous lesion. The various precursor lesions in the body include cervical intraepithelial lesions, atrophic gastritis of pernicious anemia, leukoplakia of vulva, penis or oral cavity, chronic ulcerative colitis and solar keratosis of skin. The terminology of "precancerous lesion" is not correct as many of such lesions may not develop malignancy in course of time. Precancerous lesion is often graded from I, II and III depending on the degree of nuclear atypia and relative involvement of the epithelial layer by the lesion. The essential characteristics of the precancerous lesions are:

- The cells are restricted to the epithelial layer only. There should not be any sign of invasion to the deeper tissue
- The individual cells show mild to moderate nuclear enlargement and pleomorphism
- Most of the time, these lesions are asymptomatic and a thorough search is needed to detect such lesions. Cytologists have a great role in detection of preneoplastic condition from

scraping or smear so that the lesion could be excised to block the progression of the disease
- The exact biological future of the precancerous lesion is unpredictable. Most of the benign lesions of our body do not undergo malignant change. Therefore, there is no general rule that a cancer should always pass through a precancerous condition.

MORPHOLOGY OF CANCER CELL

It is very important for a cytologist to identify the cancer cell by light microscopy. Many of the morphological characteristics of benign and malignant cells are overlapping. **Table 5.5** shows the differentiating morphological features of benign and malignant cell (**Fig. 5.24**).

Therefore, the combinations of cytomorphologic changes are important for identification of a cancer cell (**Box 5.9**).

OVERALL PATTERN

The overall pattern of the background of cancer has certain characteristic features. Cytology smear of cancer shows hemorrhagic and necrotic background. In addition tumor diathesis may also be present.

CELL AND CYTOPLASM

Cancer cell may show cytoplasmic changes. These changes may or may not be helpful in diagnosis because benign cells may also show similar features.

TABLE 5.5: Benign versus malignant cell

Features	Benign cell	Malignant cell
Nuclear size	Usually not enlarged	Nucleus enlarged and high nucleocytoplasmic ratio
Nuclear shape	Not variable	Variable-shaped nucleus
Nuclear margin	Regular	Irregular
Nucleoli: Number Size	 Single, Regular, small	 Single to multiple, Irregular, enlarged
Nuclear hyperchromasia	Normochromatic	Hyperchromatic
Chromatin texture	Fine, nondescriptive	Coarse, irregular clumped
Micronuclei	Occasionally seen	Increased number of micronuclei
Mitotic rate	Low	High
Atypical mitosis	Not present	Tripolar star-shaped atypical mitosis

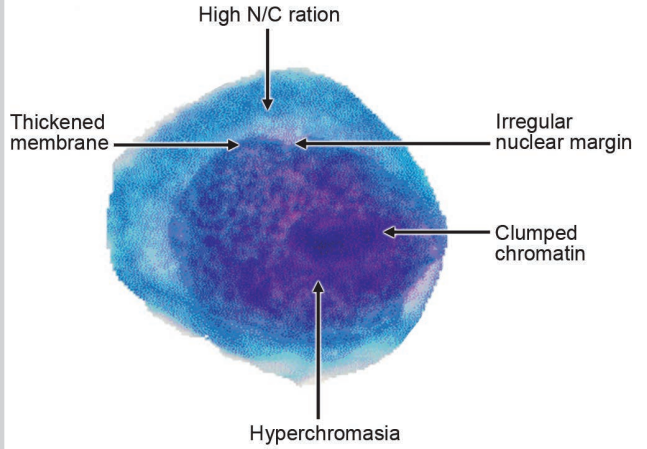


Fig. 5.24: The figure highlights morphological features of the malignant cells

BOX 5.9 **Characteristic morphological changes**

Background
- Tumor diathesis
- Blood
- Necrosis

Cell
- Enlarged cells
- Pleomorphic
- Loss of cell polarity

Nucleus
- Nuclear pleomorphism
- High nucleocytoplasmic (N/C) ratio
- Irregular and focally thickened nuclear margin
- Nuclear budding and fragmentation
- Nucleolar abnormalities: enlarged, multiple, pleomorphic
- Nuclear hyperchromasia
- Alteration of chromatin pattern: fine, reticular, irregular clumped
- Mitosis
  - Increased in number
  - Qualitative abnormality: tripolar and multipolar-shaped mitotic figures
- Other changes
  - Multinucleation
  - Intranuclear inclusions
  - Cell cannibalism
  - Excess Barr body
  - Excess micronuclei

Size

Cancer cells are usually bigger than its normal counterpart. However, this may not be true all the times. Cytoplasm of the cancer cell may not always be more in amount relative to enlargement of nuclei. Therefore, there may be gross alteration of the nucleocytoplasmic (N/C) ratio. Cancer cells

may show variation of size among themselves known as anisocytosis.

Shape

Cancer cells usually show variable shapes and even grotesque shape. Benign cells usually do not show much variation in shape. However, occasionally regenerative cells may show bizarre shapes. Shape is not a reliable criterion to identify malignant cells.

Cell Membrane and Cell-to-Cell Junction

Cell-to-cell junction is usually disrupted in cancer cell. The cell polarity is lost in epithelial malignancy. Therefore, the specific polar location of villi is not maintained. There may be variable sized microvilli over the entire surface of the cancer cell. In contrast, the benign cell maintains the polarity (epithelial) and the villi are located in one end. The size of the villi is regular in benign cells. Malignant epithelial cells undergo a process of transition, known as epithelial mesenchymal transition, which is responsible for tumor progression and metastasis. This has been discussed previously.

NUCLEUS

The major changes of a malignant cell are seen in the nucleus (**Figs 5.25A to F**).

The characteristic light microscopic changes of cancer cells are:

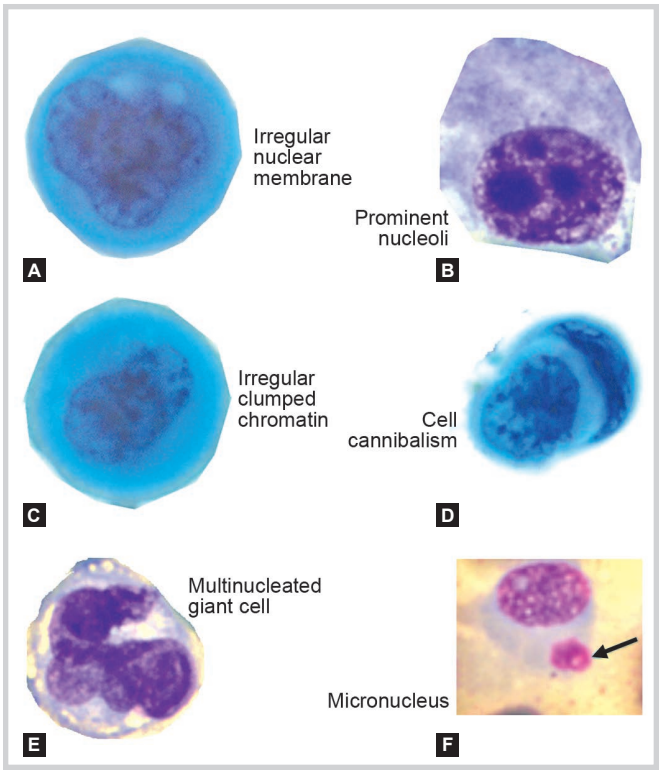
- Nuclear enlargement and pleomorphism
- Nuclear margin irregularity
- Thickening of nuclear membrane
- Nuclear budding and fragmentation
- Nucleolar abnormalities
- Nuclear hyperchromasia and change of chromatin pattern
- Mitosis
- Other changes.

Nuclear Enlargement and Pleomorphism

Malignant cell shows nuclear enlargement. As the nucleus occupies the major part of cytoplasm, so the N/C ratio is increased. The exact cause of nuclear enlargement is difficult to say. However, the possible causes are:

- Increased DNA content and aneuploidy
- Increased amount of histone and nonhistone proteins in the nucleus.

In addition, the nuclei of the malignant cells often show significant variation of size known as nuclear pleomorphism. The basis of nuclear pleomorphism has been explained by mutator phenotype theory.[85] It says that a large number of genetic mutations occur during early part of carcinogenesis. The malignant cells pick up the most suitable mutational change for progression of carcinoma. Therefore, there may be heterogeneous population of multiple subclones of cell with unique genetic mutation. These malignant cells have characteristic behavioral changes along with variation of nuclear shape and size.



Figs 5.25A to F: Microphotograph highlights various nuclear features of malignant cells. (A) Irregular nuclear membrane is often seen in cancer cell; (B) Multiple large pleomorphic nucleoli are one of the characteristic features of cancer cell; (C) Irregular clumped chromatin is the most reliable sign of malignant cell; (D) Malignant cell often engulfs another malignant cell known as cannibalism; (E) Multinucleated tumor giant cell is seen in various types of malignancies; (F) Micronucleus is the small fragment of nucleus that can be easily identified on routine Giemsa stain

Nuclear Margin Irregularity

Nuclear contour irregularity is often noted in the malignant cell. There may be nuclear cleaving, convolution or molding. The abnormality of nuclear membrane may be subtle in the form of nuclear protrusion or notch. These are difficult to detect and careful observation is needed for identification of membrane abnormalities. The factors responsible for change of nuclear contour are (**Box 5.10**):

- Quantitative or qualitative changes of lamin: There is adequate evidence that qualitative or quantitative changes of nuclear lamin of nuclear envelope are responsible for alteration of nuclear margin and shape.[86] If the lamin protein is biochemically knocked out then the nuclei become small and fragile.[87]

  In fact, both qualitative and quantitative changes of lamin have been demonstrated in small cell carcinoma of lung with nuclear molding, colonic carcinoma, and gastric carcinomas.[88,89]

BOX 5.10 Nuclear margin irregularity

Nuclear cleaving, convolution or molding noted in cancer cell
Probable causes:
- Quantitative or qualitative changes of lamin: Lamin is altered in different cancers
- Gene silencing by Rb-protein: pRB binds with inner nuclear membrane proteins and causes conformational changes of chromatin and silencing of gene
- RET-PTC oncogenic changes
- Nuclear matrix protein: Alteration of nuclear matrix protein in various cancers

- Gene silencing by retinoblastoma-protein: pRB binds with inner nuclear membrane protein and other factors such as histone deacetylase, DNA methyl transferases, histone methyl transferases, and heterochromatin protein 1. This protein complex is responsible for change of conformation of chromatin structure and silencing of gene leading to carcinogenesis. Therefore, membrane abnormality is expected in cancer nuclei.[91]
- Rearranged in transformation/papillary thyroid carcinoma (RET-PTC) oncogenic changes: RET-PTC oncogenic transformation is frequently noted in papillary thyroid carcinoma with nuclear grooves. However, the exact cause and effect of relationship of RET-PTC oncogenic transformation and nuclear groove is not known so far.[92]
- Nuclear matrix, nuclear shape and tumor: An alteration of nuclear shape may occur due to change in the composition of the nuclear matrix protein, the internal skeleton of the nucleus.[87] Nuclear matrix protein is tissue and cancer specific and its composition varies depending on nuclear activities.

Thickening of Nuclear Membrane

Thickening of nuclear membrane is often seen in cancer nuclei. This is due to the peripheral heterochromatin formation in the nuclear periphery of the malignant nuclei.

Nuclear Budding or Fragmentation

Nuclei of the malignant cells often show increased fragmentation or budding of nuclei, ring-shaped nuclei and nuclear holes.

Nucleoli

Nucleoli of the malignant cell are often multiple, enlarged, and pleomorphic. The nucleoli are easily detectable by light microscope. In hematoxylin and eosin-stained histological section, the nucleoli are stained as deep eosinophilic round structures and in May Grunwald Giemsa (MGG)-stained cytology smears, the nucleoli are stained as light blue-colored structures (**Figs 5.25A to F**). Nucleoli are related with ribosome synthesis. The proliferating cells have increased demand for protein synthesis, and therefore need increased rate of ribosomal biogenesis.[92] As the cancer cells have higher growth fraction and metabolic activity, so it is expected that the cancer cells show enlarged nucleoli. However, the cell proliferation rate may not be the sole factor of nucleolar change. Several tumor-suppressor gene products particularly p53 and pRb accumulate in nucleoli and lead to increased rRNA transcriptional activity. Alteration of the pathway of p53 and pRb in cancer cell may be directly related with nucleolar changes.[93]

Nuclear Chromatin

Nuclei of the cancer cell are usually dark colored known as hyperchromatic. The dark color of the nucleus may be due to increased amount of DNA, increased histone, and nonhistone proteins. Changes of chromatin pattern of nucleus are the hallmark of cancer cell. Cancer nuclei may show salt-and-pepper chromatin, opened up reticular chromatin, irregular coarse clumped chromatin, and vesicular chromatin (**Fig. 5.25B**). Fine chromatin pattern is seen in leukemia, adenocarcinoma and many lymphoma cases (**Figs 5.26A to D**). Salt and pepper chromatin is noted in nuclei of neuroendocrine tumor and carcinoid. Nuclei of lymphoblastic lymphoma show reticular chromatin. Any high-grade carcinoma shows irregular clumped chromatin (**Fig. 5.26D**).

The exact cause of abnormal chromatin pattern is still not clearly known. However, the possible factors responsible for the alteration of chromatin pattern are (**Box 5.11**): (1) chromatin relocation, (2) chromatin remodeling, (3) DNA aneuploidy, (4) change of nuclear matrix protein, and (5) change of nuclear pore.[94]

Chromatin

1. Chromatin relocation

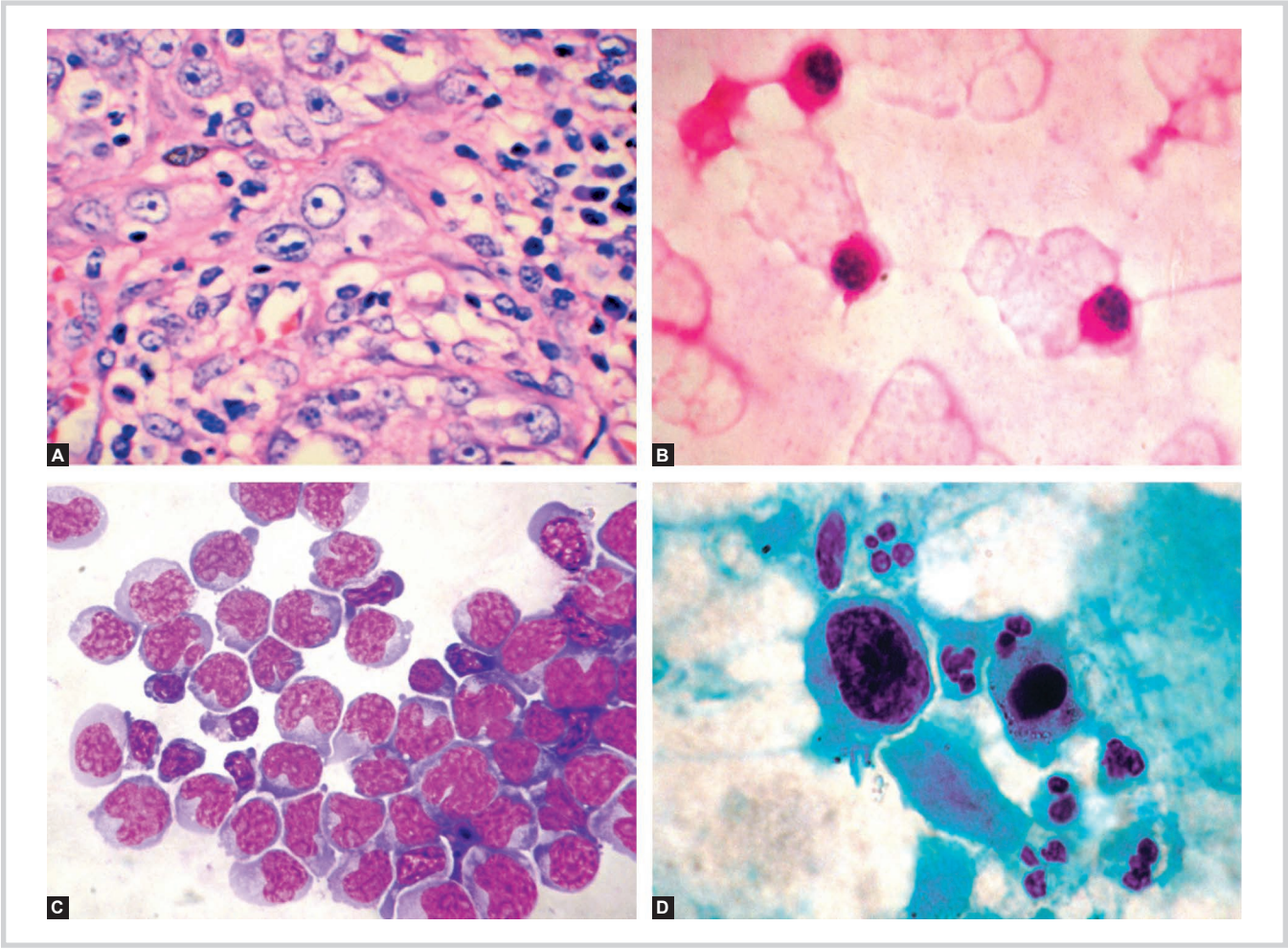
Each chromosome occupies a specific position in the nucleus. The gene poor chromosome occupies in the peripheral part of the nucleus, whereas the gene rich chromosomes are located in the central part of the nucleus. The malignant cells often show increased gene expression, and therefore, the chromosomal location may be altered. This relocation of chromosome may be responsible for chromatin pattern alteration.

2. Chromatin Remodeling

There is a significant chromatin remodeling during the activation or repression of genes. Acetylation of histones causes opened up chromatin followed by gene expression, whereas deacetylation of histone represses the gene activity. Histone acetylation and deacetylation is a dynamic process. In case of various malignancies, the alteration of histone acetylation has been noted.[95] The altered histone acetylation may be responsible for conformational change chromosome followed by change of chromatin pattern.

3. Deoxyribonucleic Acid Aneuploidy

Aneuploidy or abnormal DNA content of the cancer cell may be responsible for altered packing of the chromatin and changes of chromatin texture.



Figs 5.26A to D: Microphotograph highlights different types of chromatin pattern. (A) Vesicular clear chromatin in adenocarcinoma uterus (hematoxylin and eosin X MP), (B) Cartwheel-like chromatin pattern in plasma cells uterus (hematoxylin and eosin X OI), (C) Fine chromatin in acute lymphatic leukemia (May Grunwald Giemsa X OI), (D) Irregular clumped chromatin in squamous cell carcinoma of cervix (Papanicolaou's stain X OI)

BOX 5.11 **Nuclear chromatin**

Change of chromatin pattern is the hallmark of cancer cell
- Different pattern:
  - Salt-and-pepper: Neuroendocrine tumor and carcinoid
  - Fine chromatin pattern: Leukemia, adenocarcinoma, lymphoma cases
  - Reticular chromatin: Lymphoblastic lymphoma
  - Irregular clumped chromatin: Any high grade carcinoma
- Etiology:
  - Chromatin relocation
  - Chromatin remodeling
  - DNA aneuploidy
  - Change of nuclear matrix protein
  - Change of nuclear pore

4. Nuclear Pore

Nuclear pore helps in the transmission of various cytoplasmic contents into the nucleus and *vice versa*. The abnormal diffusion of various materials may affect the nuclear chromatin pattern.

MITOSIS

Abnormality in mitosis may be either quantitative or qualitative.

Quantitative Abnormality

Cell proliferation is one of the important hallmarks of cancer. This is reflected as increased number of mitosis in the tumor tissue. Mitotic phenomenon is also noted in case of proliferating cells of regenerative tissue. However, the rate of mitosis is almost

equal to death rate of the cells in proliferating tissue, and the mitotic phenomenon is a temporary event.

Qualitative Abnormality of Mitosis

Qualitative abnormality of the mitotic phenomenon is an important characteristic of cancer. Instead of bipolar mitotic figure, there may be tripolar, multipolar or incomplete multipolar mitotic figures. The various causes of mitotic abnormality and ChIN have been described in detail in Chapter 4.

OTHER NUCLEAR CHANGES

Various other nuclear changes are often associated with malignancy, such as:

Multinucleation

Multinucleated cells may be seen in malignancy (**Figs 5.25A to F**). However, the multinucleated cells can also be seen in benign tumors and in chronic inflammation. So, it is a nonspecific feature.

Intranuclear Inclusion

Intranuclear pseudoinclusions represent an invagination of the cytoplasm into the nucleus (**Fig. 5.27**). The intranuclear inclusions (INI) are generally easier to appreciate in cytological preparations than frozen sections or histopathology sections. It appears as round to oval pale structure within the nucleus. It looks like a pink-colored round structure in MGG-stained smears and a grayish to light-greenish appearance in Papanicolaou-stained material.[96] Electron microscopic study clearly shows that the INI is of cytoplasmic origin and contains cytoplasmic structures, such as mitochondria, premelanosomes, melanosomes, microfilaments, ribosomes, rough endoplasmic reticulum, and annulate lamellae. Intranuclear inclusions are separated from the nuclear matrix by a double-layered membrane.[97] Intranuclear inclusions are commonly seen in papillary thyroid carcinoma, malignant melanoma, papillary renal cell carcinoma, choroid plexus carcinoma, and human ovarian surface epithelial cells.[96] However, benign lesions, such as meningioma or lymphocytic thyroiditis may also show INIs. The exact pathogenesis of the INI is unknown. The probable causes of INIs are:

- Altered ratio of nuclear surface to volume: Leduc and Wilson suggested that that INI develops as an attempt to maintain the normal ratio of nuclear surface to nuclear volume.[98]
- Swollen excess cytoplasm: Sobel et al. consider that the majority of inclusions can be explained by the swollen cytoplasm extruding into the nucleus[99]
- Nuclear grooves followed by inclusion: It may also be possible that the infolding of nuclear membrane is the early stage of the deep cytoplasmic invaginations that are responsible for the formation of the INIs[100]

Excess Barr Bodies or Sex Chromatin

Barr body is the inactive X chromosome in a female somatic cell. It is readily identified as planoconvex structure of 2–3 microns in diameter on the periphery of the nuclear membrane. Number of Barr body in a nucleus is less than one of the total numbers of X chromosomes. This means female with two X chromosome will have one Barr body. However, the malignant cell shows more than one Barr body. This is an interesting finding in cancer cell.

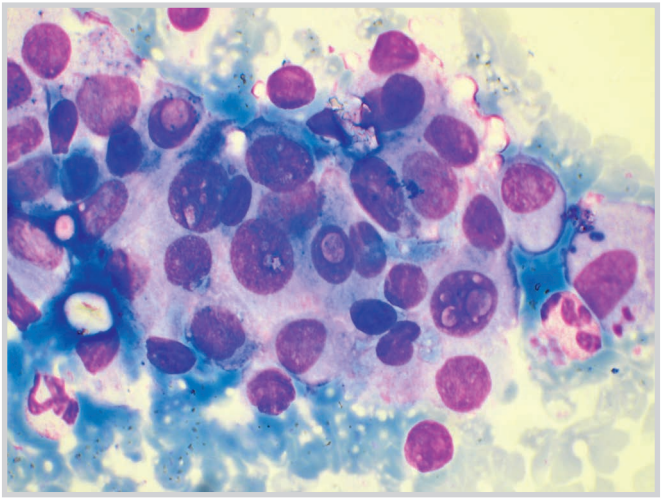


Fig. 5.27: Multiple intranuclear cytoplasmic pseudoinclusions in papillary carcinoma of thyroid (May Grunwald Giemsa X OI)

Cell Cannibalism

Cellular cannibalism, defined as a large cell enclosing a slightly smaller one within its cytoplasm.[101] The cannibalistic cell is composed of a crescent-shaped nucleus engulfing another cell with round to oval-faded nucleus (**Fig. 5.25 D**). The cannibalistic cells have the unique property to engulf the fellow tumor cells. The cannibalized cell remains alive within the cannibalistic cell at a certain period of time. Cannibalism is different than phagocytosis, entosis, emperipolesis, and autophagy. Cell cannibalism is an important morphologic feature to distinguish benign from a malignant lesion and has been described in various cancers such as transitional cell carcinoma of bladder, breast cancer, lung carcinomas, etc. and this is related with the aggressiveness of the malignancy.

Excess Micronuclei

A micronucleus (MN) is a small additional nucleus readily identifiable by light microscopy as nonrefractile, round to oval-shaped structure with a smooth perimeter suggestive of a membrane. The diameter of MN is variable from one-sixteenth to one-third the diameter of the main nucleus and the shape, color, and texture of MN are similar to that of nucleus (**Fig. 5.25 F**). Micronucleus can be demonstrated on simple MGG stain or Papanicolaou's stain. However, specific DNA stain such as Feulgen stain or Acridine orange stain can be used to demonstrate it. Micronucleus is developed by chromosomes or chromosome segments that fail to be incorporated in cell nuclei during cell division. The formation of MN represents a measure of both chromosome breakage as well as chromosome loss.[102] Micronucleus has often been used as a biomarker of

chromosomal damage, genome instability and cancer risk. The number of MN is usually increased in various malignancies.

CHARACTERIZATION OF TYPE OF CANCER CELL

After the initial diagnosis of malignancy in cytology, it is often necessary to characterize the type of malignancy. As described previously, the malignant cells usually try to simulate the tissue of origin. The cancer cells either morphologically mimic the cell of origin, or they produce the substance derived from the parental tissue.

Table 5.6 shows the various examples of morphological resemblance of malignant cells with the parental tissue. The duty of the cytologist is to find out these features for exact subtyping of the cancer. At times, the products of the cancer cells are also important (**Table 5.6**), such as keratin in squamous cell carcinoma, mucin in adenocarcinoma, osteoid in osteosarcoma, cartilaginous material in chondrosarcoma, melanin in melanoma, etc.

Ancillary Tests

At times, it is necessary to have further confirmation of subtype of cancer. In fact, in poorly differentiated malignancy, it is very difficult to categorize the tumor by morphology alone. In these circumstances, ancillary tests are needed (**Table 5.7**). Immunocytochemistry may be very helpful for exact subtyping in most of the cases.

DIAGNOSTIC PITFALLS OF MALIGNANCY

There are various mimickers of malignant cells (**Box 5.12**). The cytologist should carefully assess this condition. Enlarged nucleus, mild pleomorphism, and prominent nucleoli are the main misleading features. Cytologist should give more importance on nuclear chromatin pattern, nucleocytoplasmic ratio, and overall cytological features. Clinical histories of the individual cases are also very important factor to avoid false-positive cases.

TABLE 5 6: Morphological resemblance and products of cancer cell

Malignancy	*Morphological features*	*Products*
Squamous cell carcinoma	Polyhedral squamoid cells with eosinophilic cytoplasm, keratin pears	Keratin
Adenocarcinoma	Columnar looking cells with eccentric nuclei and vacuolated cytoplasm	Mucin
Malignant melanoma	Round to oval pleomorphic cell with intracytoplasmic melanin	Intra and extracellular melanin
Hepatocellular carcinoma	Polyhedral hepatocyte-like cells	Cells may contain bile pigment
Osteosarcoma	Cells look like osteoblasts	Osteoid
Chondrosarcoma	Cells look like chondrocytes	Cartilaginous material

TABLE 5.7: Ancillary immunological tests for broad categorizations of cancer

Malignancy	*Immunocytochemistry*
Carcinoma	Epithelial membrane antigen, cytokeratin
Lymphoma	CD45 (leukocyte common antigen)
Melanoma	HMB45, Mela A
Hepatocellular carcinoma	Hep Per 1, Glyciphan 3
Sarcoma	Vimentin
Rhabdomyosarcoma	Desmin
Leiomyosarcoma	Smooth muscle actin
Nerve sheath tumor	S-100
Neuroendocrine tumor	Chromogranin, neuron-specific enolase

BOX 5.12 Potential pitfalls in diagnosis of cancer

False-positive:
- Inflammation: Inflammatory atypia in bacterial or viral infections
- Chemotherapy and radiotherapy-induced changes
- Reactive changes: Reactive mesothelial hyperplasia, lithiasis, etc.
- Benign neoplasm showing nuclear pleomorphism: Paraganglioma, Schwannoma, pleomorphic lipoma, etc.
- Metaplasia: Endometriosis

False-negative:
Malignancy with monomorphic nuclei: Lobular carcinoma of breast, low grade non-Hodgkins lymphoma, follicular carcinoma, etc.

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CHAPTER 6

Tissue and Cell Organization

Chapter Contents

There are approximately two hundred distinct cell types in our body. These cells are arranged and organized to form tissues. Different types of tissues are organized to form a complete organ. The cytologist's prime duty is to identify the tissue types of cells and then the basic character of the cell (benign versus malignant).

The four basic tissue types are: epithelial, connective, muscle, and neural tissue.

EPITHELIAL TISSUE

Classification of epithelial tissues is based on:

- The number of the constituent cell layer, such as single layer of epithelium is known as simple epithelium, whereas the multiple layer of epithelium is known as stratified epithelium
- Individual cell morphology, such as flattened cell, columnar cell or cuboidal cell and
- The presence of surface specializations, such as cilia or keratin.

Classification of Epithelial Tissue

- Simple
  - Squamous
  - Cuboidal
  - Columnar
- Pseudostratified
- Stratified
  - Squamous keratinized
  - Squamous nonkeratinized
  - Cuboidal
  - Columnar
  - Transitional.

Simple Squamous Epithelium

This is defined as the surface epithelium with single layer of squamous cells. The lining of pulmonary alveoli, loop of Henle, parietal layer of Bowman capsule and the delicate lining of the pleural, pericardial and peritoneal cavities are formed by simple squamous epithelium (**Fig. 6.1**).

Simple Cuboidal Epithelium

It is composed of a single layer of cuboidal cells. When the epithelial cells are viewed in cut section or smears, the cells present a square profile with centrally placed round nuclei. The simple cuboidal epithelium forms the lining of many small ducts, kidney collecting tubules and ovarian surface (**Fig. 6.2**).

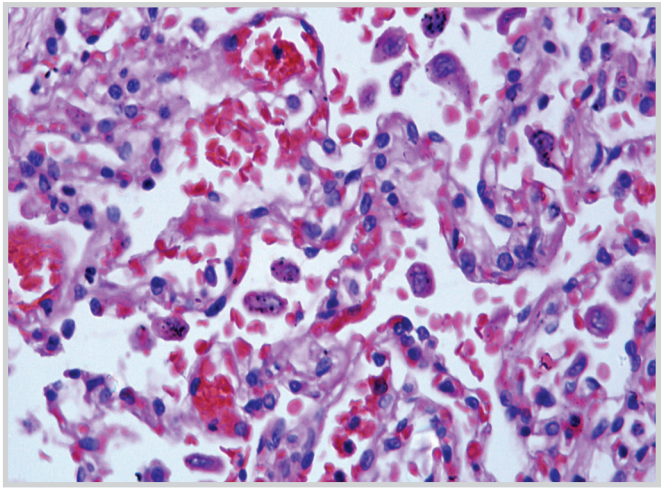

Fig. 6.1: Simple squamous epithelial cell on the lining of pulmonary alveoli (Hematoxylin and eosin stain X HP)

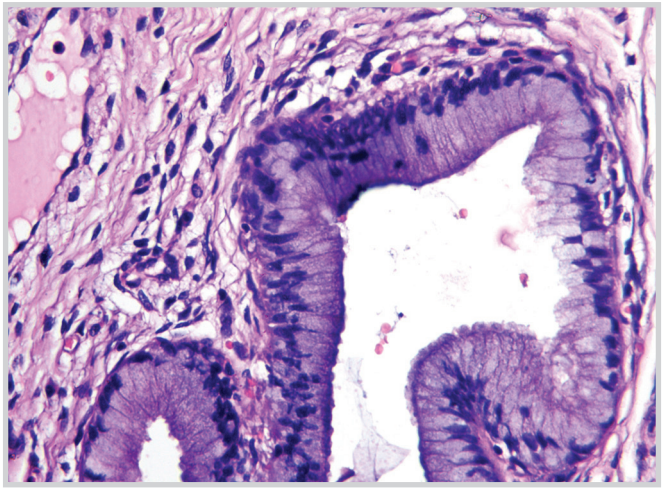

Fig. 6.3: Simple columnar epithelial cell on the lining of endocervical gland (Hematoxylin and eosin stain X HP)

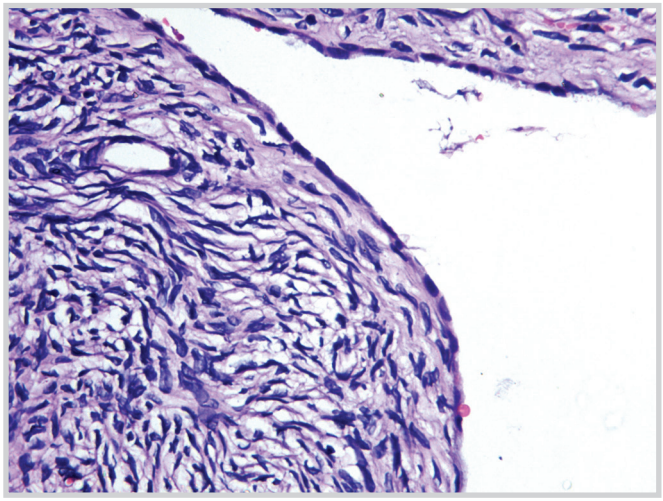

Fig. 6.2: Simple cuboidal epithelial cell of the lining of ovarian surface (Hematoxylin and eosin stain X MP)

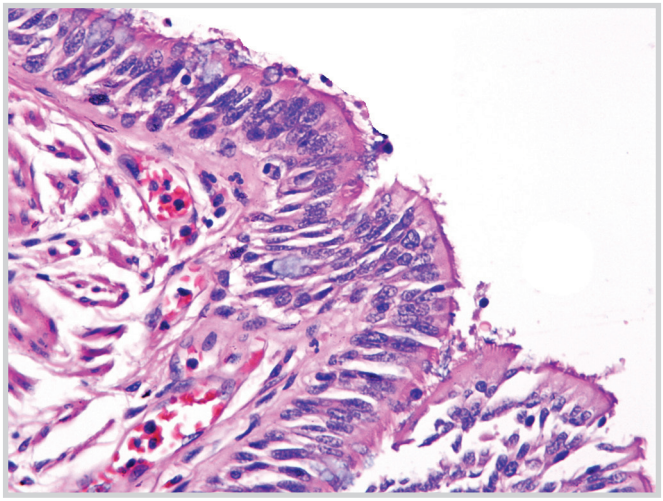

Fig. 6.4: Pseudostratified columnar epithelium of bronchial lining cells (Hematoxylin and eosin stain X MP)

Simple Columnar Epithelium

Simple columnar epithelium is composed of a single layer of tall columnar epithelial cells. In longitudinal view, the cells look like tall with basally placed nuclei. Simple columnar epithelium forms the lining of the intestine, gall bladder and large ducts or glands (**Fig. 6.3**). The epithelial cells often show surface microvilli or cilia.

Pseudostratified Columnar Epithelium

As the name indicates, the cells appear as stratified but actually are present in single layer. All the cells are attached to the basal lamina but only some cells reach to the free surface of the membrane. The cells are columnar looking and many of them show cilia on their apical surface (**Fig. 6.4**). The lining of most of the trachea, primary bronchi, epididymis, ductus difference, auditory tube, part of the tympanic cavity, the nasal cavity, lacrimal sac and male urethra show pseudostratified-ciliated columnar epithelium.

Stratified Squamous Epithelium

Stratified squamous epithelium is comprised of several layers of epithelial cells. Only the cells of basal layer are in contact with basement membrane. These basal cells are cuboidal in shape. The cells located in the intermediate portion are more polyhedral and the cells on the surface layer are flattened in shape. This is found in the lining of mouth, epiglottis, esophagus, vocal folds and the cervix (**Fig. 6.5**).

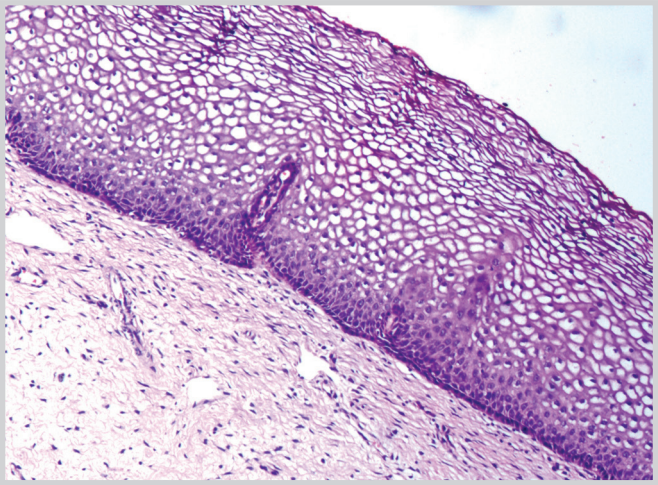

Fig. 6.5: Nonkeratinized stratified squamous epithelium in ectocervix (Hematoxylin and eosin stain X MP)

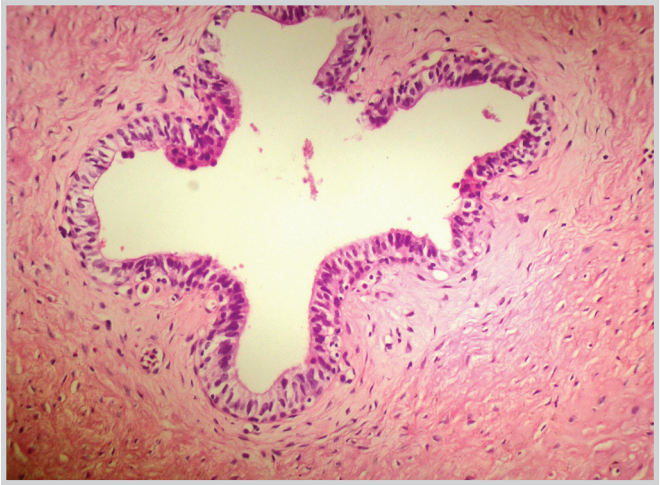

Fig. 6.7: Transitional lining epithelium in ureter (Hematoxylin and eosin stain X LP)

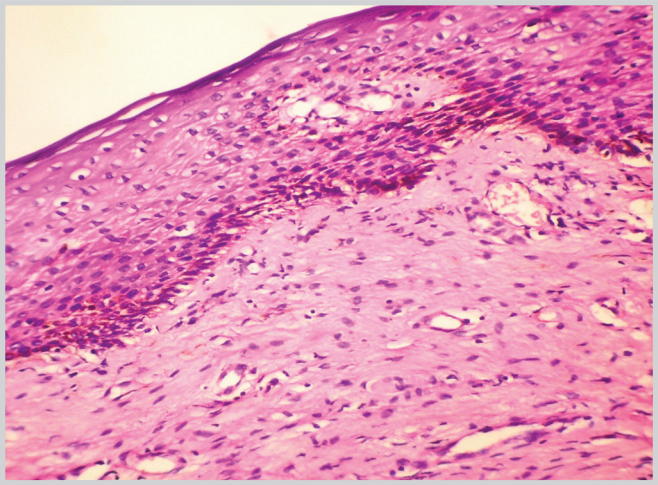

Fig. 6.6: Keratinized stratified squamous epithelium in skin (Hematoxylin and eosin stain X MP)

In case of keratinizing stratified squamous epithelium, the cells in the superficial layers are dead and replaced by keratin. This is found in the epidermal layer of skin (**Fig. 6.6**).

Stratified Cuboidal Epithelium

The stratified cuboidal epithelium is comprised of only two layers of cuboidal cells. It lines the large duct of sweat glands.

Stratified Columnar Epithelium

Stratified columnar epithelium contains more than one layer of cells. The cells adjacent to the basal lamina are cuboidal and superficial layer of cells are columnar in appearance. The lining of the conjunctiva of the eye and large excretory ducts are composed of stratified columnar epithelium.

Transitional Epithelium

Transitional epithelium composed of multiple layers of cells. The basal cells are cuboidal or columnar and the intermediate groups of cells are polyhedral in shape. The cells on the top are dome-shaped with bulge into the luminal side. The linings of almost all the parts of the urinary system such as renal pelvis, ureter, bladder and urethra are made up of transitional epithelium (**Fig. 6.7**).

The brief description and locations of different types of epithelial cells are listed in **Table 6.1** and schematic cytomorphology of different type of epithelium has been highlighted in **Figure 6.8**.

CONNECTIVE TISSUE

Connective tissue connects different tissues in our body. Mature connective tissue is classified as:

- Connective tissue proper
  - Loose areolar tissue
  - Dense connective tissue
- Specialized connective tissue
  - Adipose tissue
  - Hematopoietic tissue
  - Bone
  - Cartilage

Major cells in the connective tissue are divided into:

- Fixed Cells
  - Fibroblasts
  - Adipose cells
  - Macrophages
  - Pericytes
  - Mast cells

TABLE 6.1: Different types of epithelium

Type of epithelium	*Description*	*Location*
Simple		
Squamoid	Single layer of flattened cells	Lining of pulmonary alveoli, loop of Henle, parietal layer of Bowman capsule and delicate lining of the pleural, pericardial and peritoneal cavities
Cuboidal	Single layer of cuboidal cells	Lining of many small ducts, kidney collecting tubules and ovarian surface
Columnar	Single layer of columnar cells	Lining of the intestine, gall bladder and large ducts or glands
Pseudostratified	Single layer of cells. All the cells are attached with the basal lamina but only some cells reach to the free surface of the membrane.	Lining of most of the trachea, primary bronchi, epididymis, ductus difference, the auditory tube, part of the tympanic cavity, the nasal cavity, lacrimal sac and male urethra
Stratified		
Nonkeratinized squamous epithelium	Multiple layers of epithelial cells. The basal cuboidal cells to intermediate polyhedral cells to superficial flattened cells	Lining of mouth, epiglottis, esophagus, vocal folds, vagina
Keratinized squamous epithelium	Same as above but the cells in the superficial layers are dead and replaced by keratin	Epidermis of skin
Cuboidal	Only two layers of cuboidal cells	Lining of the large duct of sweat glands
Columnar	Multiple layers of cells. The cells adjacent to basal lamina are cuboidal and superficial layer of cells are columnar in appearance	Lining of conjunctiva of the eye and large excretory ducts
Transitional	Multiple layers of cells, basal superficial cells to polyhedral to dome-shaped superficial cells	Lining of renal pelvis, ureter, bladder and urethra

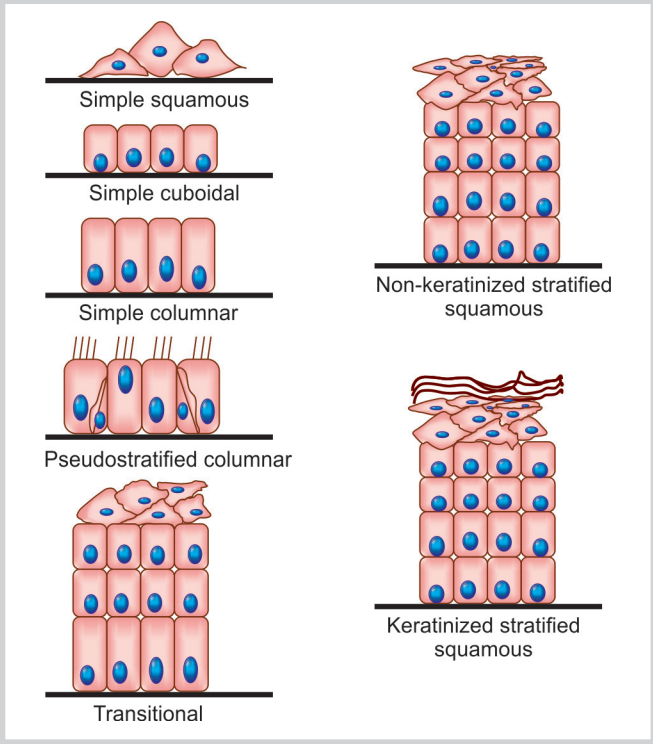

Fig. 6.8: Schematic diagram of different types of epithelial cells

- Transient cells
  - Plasma cells
  - Lymphocytes
  - Neutrophils
  - Monocytes
  - Eosinophils
  - Basophils.

Fixed Cells

Fibroblast

These cells may present in the active or inactive form as fibrocyte. Fibroblast is oval to elongated cells with ample amount of cytoplasm. Nuclei of the fibroblasts are oval to elongated with single prominent nucleoli. In contrast, inactive fibroblast or fibrocyte is smaller with eosinophilic cytoplasm and elongated nuclei.

Myofibroblasts are modified fibroblasts with properties of both fibroblast and smooth muscle.

Adipose Cells

The fat cells or adipocytes are large cells with abundant vacuolated cytoplasm having eccentric nuclei. The cells often look like signet ring cells.

Macrophages

Macrophages are the normal constituents of the connective tissue. These are large cells with abundant amount of vacuolated cytoplasm. The nucleus is central to eccentric, ovoid to kidney bean in shape with condensed chromatin. The nucleolus is inconspicuous. The cytoplasm of the macrophages often contains phagocytosed debris.

Pericytes

These cells are arranged around the endothelial cells of capillaries and small venules. Pericytes are pale staining cells with moderate cytoplasm and oval to elongated nuclei.

Mast Cells

Mast cells are derived from the bone marrow stem cells. They are large cells with a centrally placed round to ovoid nuclei. The cytoplasm of the mast cell contains abundant metachromatic blotchy granules comprised of histamines, heparin, aryl sulfatase and neutral proteases.

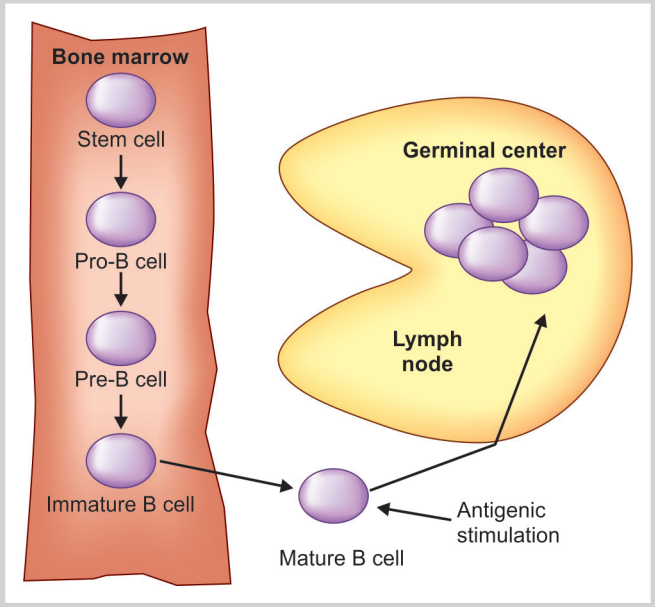

Fig. 6.9: Schematic diagram shows development of B lymphoid cells

Transient Cells

Plasma Cells

Plasma cells are scattered throughout the connective tissue and they are often concentrated in chronic inflammation. These cells are round to oval cells with intensely basophilic cytoplasm and eccentric nuclei. Nucleus of the cell has peripheral clumped chromatin giving a cart wheel appearance.

Lymphocytes

Lymphocytes are originally present in blood. They accumulate in the connective tissue during chronic inflammation from blood. The lymphocytes are heterogeneous population of cells with great difference in origin, lifespan, preferred location in the lymphoid organs, surface immunophenotyping and function. Morphology of lymphocytes provides no clue to their immunophenotying features.

Morphology

Lymphocytes are small round cells with 6–9 microns in diameter. They have scanty deep basophilic rim of cytoplasm. The nucleus is round with condensed chromatin and inconspicuous nucleoli. Large lymphocytes are a bit larger in size and are also known as large granular lymphocytes as they possess azurophilic granules in the cytoplasm.

Lymphocytes are principally classified as B lymphocyte, T lymphocyte and Null cell.

B Lymphocyte

Figure 6.9 depicts the ontogenic differentiation of B lymphoid cells. B lymphocytes develop from pluripotent stem cells of bone marrow. From the stem cells successive development of Pro-B cell, pre-B cell and immature B cell occur. From the immature B cells, mature B cell develops and comes to the circulation. These mature naïve B cells migrate to the outer region of the lymph node in the primary follicles. When the mature naïve B cells encounter with antigen, they migrate to the T-cell zone of the lymph node and transform into large B blast. The antigen-primed B cells further migrate into the primary follicles. Here, they proliferate and differentiate into centroblasts to form the germinal center. Each B lymphocyte is destined to generate only one specific antibody or immunoglobulin. Immunoglobulin has two major components: heavy chains and light chain. There are five types of heavy chains: alpha, beta, gamma, delta and epsilon. Similarly, there are two types of light chains kappa and lambda. Each immunoglobulin contains only one type of heavy chain and light chain. There are five isotypes of immunoglobulin IgM, IgA, IgG, IgD and IgE. During the development of B lymphoid cells in the bone marrow, a series of gene rearrangement happen known as V, D, J gene rearrangement. With the help of this VDJ gene rearrangement a unique immunoglobulin is formed. VDJ gene rearrangement is initiated from the pre-B cell.

T Lymphocytes

T lymphocytes are small round to oval cell with scanty light blue cytoplasm having round nuclei with condensed chromatin. Morphologically, they are indistinguishable from B lymphocytes. T lymphocytes initially develop in the bone marrow and then migrate to thymus to become immunocompetent. T lymphocytes contain surface receptor known as T-cell receptor (TCR). The constant portion of TCR is attached with the membrane and the variable amino-terminal region that binds with the antigen is extending away from the membrane. T-cell receptor makes a complex with other membrane proteins CD4, CD8, and CD3 and thus making a TCR complex.

T lymphocytes have several subtypes based on the surface molecules and functions:

Helper-T cells: T helper (Th) cells show CD4 protein on their surface. They work in association with antigen presenting cells and help in the maturation of B cells into plasma cells and memory cells.

Cytotoxic-T cell: T cytotoxic (Tc) cells show CD8 protein on their surface and are responsible for killing virally infected cells, foreign cells and tumor cells.

Memory T cell: Memory T cells are responsible for long-term memory of the immune system. These cells quickly expand to large number of effector cells after reexposure to the antigen. These cells show cell surface protein CD45RO and they are either CD4 or CD8 positive.

T regulatory cell: T regulatory cells possess CD4 molecules on the surface and related to immunosuppression so they were previously known as T suppressor cells. There are two types of T_{reg} cells:

1. Naturally occurring T_{reg} cells: These cells develop in thymus and when they interact with antigen presenting cell, they suppress the immune response in a nonantigen-specific manner. Naturally occurring T_{reg} cells show characteristic intracellular FoxP3.
2. Adaptive T_{reg} cells: They are also known as Th3 cells. These cells develop from naïve T cells.

Natural killer T cells: Natural killer T (NKT) cells are the effector T cells that resemble NK cells. Natural killer T cells recognize glycolipid antigen presented to them in conjunction with CD1d molecules. Activated NKT cells can function as both Th and Tc cells (**Box 6.1**).

BOX 6.1 **Types of T cells**

- Helper T cells: CD4 protein on cell surface
- Cytotoxic T cell: CD8 protein on their surface
- Memory T cell: CD45RO and either CD4 or CD8 positive
- T regulatory cell:
  - Naturally occurring T_{reg} cells: Contains intracellular FoxP3
  - Adaptive T_{reg} cells: Developed from naïve T cells
- Natural killer T cells: Recognize glycolipid antigen presented to them in conjunction with CD1d molecules

CLUSTER OF DIFFERENTIATION

Cluster of differentiation commonly abbreviated as CD markers are special molecules present on the surface of the cells of our body.[1] These CD molecules are also expressed on the cell surface during various stages of differentiation of white blood cells. Cluster of differentiation nomenclature was first proposed in the human leukocyte differentiation antigen workshops for the standardization and recognition of many such molecules with important functions. This system has standardized the various monoclonal antibodies that are developed against the epitopes of the surface molecules of white blood cells in many laboratories. Since the first workshop held in Paris in 1984, about 326 CD markers have been enlisted so far. Presently CD markers have been used widely for cell type identification by immunocytochemistry or flow cytometric immunophenotyping. **Table 6.2** highlights CD expression in different types of lymphoid cells.

TABLE 6.2: T-cell cluster of differentiation markers

Cell type	*CD markers*
T lymphocytes	
All T lymphocyte	CD45, CD3,
T-helper	CD45, CD3, CD4
T-cytotoxic	CD45, CD3, CD8
T regulator	CD45, CD3, CD25, CD4
T memory	CD45, CD3, CD44, CD4/CD8
B lymphocytes	
All B lymphocytes	CD45, CD19,CD20
Pro B cell	CD34, CD10, CD19
Pre B cell	CD10, CD19, CD20
Immature B cell	CD19, CD20, CD21, CD22
Plasma cell	CD138
Macrophages	CD14, CD68
Natural killer cell	CD16, CD56

Neutrophils

They are multilobated nucleated cells with moderate amount of cytoplasm. These cells accumulate in the tissue during acute inflammation.

Eosinophils

Eosinophils show multilobated nucleus with cytoplasmic eosinophilic granules. They accumulate in tissue during allergic reaction or parasitic infection.

Basophils

Basophils contain blotchy cytoplasmic metachromatic granules. They contain heparin in the cytoplasm.

Monocytes

The cells have been described in macrophages.

REFERENCE

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SECTION 2

Clinical Cytology (Exfoliative Cytology)

CHAPTER 7

Cervical Smear: Normal Cytology, Bethesda System and Non-neoplastic Lesions

Chapter Contents

NORMAL ANATOMY, HISTOLOGY AND CYTOLOGY OF FEMALE GENITAL TRACT

Normal female genital tract is made of the following components:

- Outer vulva
- Vagina
- Cervix
- Uterus
- Fallopian tubes
- Ovaries

Figure 7.1 shows the different parts of the female genital tract.

Vulva

It is the outermost part of the female genital tract and is composed of labia majora and labia minora. Labia majora are the larger soft folds of the outer part of vulva and labia minora are the two smaller inner folds of the vulva. Clitoris, the rudimentary counterpart of penis, is the small erectile tissue situated in the anterior twofolds of labia minora.

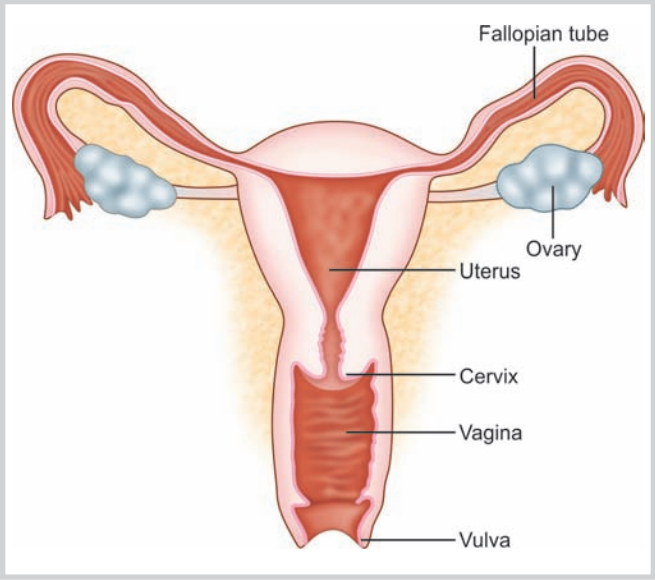


Fig. 7.1: The schematic diagram shows the different parts of the female genital tract

Vagina

Vagina is 8–9 cm fibromuscular tubal structure that joins the uterus and the outer vulva. The opening of the vagina is caudal to the urethral opening in between the labia minora. The upper part of the vagina is continuous with the cervical canal. The free space between the upper part of vaginal pouch and cervix is known as posterior vaginal fornix. This is a potential space and cells are usually accumulated here. Urinary bladder is located anterior to vagina and rectum is located posterior to vagina.

Histology

Vaginal wall has three layers: (1) inner mucosal lining made of nonkeratinizing stratified squamous epithelium, (2) muscular layer, and (3) outermost connective tissue layer. The epithelial cells of vaginal mucosa are stimulated by estrogen and contain large amount of glycogen. This glycogen serves as a nutrient for the naturally occurring bacterial flora of the genital tract. Lactic acid is released as metabolic byproduct of the utilization of glycogen that lowers the pH of that region. Low pH helps to protect from pathogenic invasion.

Uterus

This is the pear-shaped thick muscular organ measuring 7 cm long, 4 cm wide and 2.5 cm thick. Uterus has three parts: fundus, body and cervix.

Fundus

This is the upper most part of the uterus situated above the opening of the fallopian tubes.

Body

This is the central board portion where the two fallopian tubes open laterally.

Histology

Body and Fundus

The wall of the body and fundus of uterus has three layers: (1) inner endometrium, (2) myometrium and (3) outer serosa.

Endometrium

Endometrium is the mucosal lining of the uterus and consists of two layers:

1. Outer functionalis layer: This layer is the superficial layer and is shedded regularly during menstruation.
2. Inner basalis layer: This is the deeper layer from which the glands and connective tissue elements proliferate after menstrual shedding.

The mucosal lining of the endometrium is made of secretory columnar cells. Lamina propria contains many tubular glands and arterioles.

Myometrium

It is composed of three layers of smooth muscles: inner longitudinal, middle circular and outer longitudinal.

Serosa

The outer part of the uterus is lined by mesothelial cells.

Cervix

This is the lowermost circular portion of the uterus that opens into vagina. The cervix has two parts: (1) ectocervix and (2) endocervix. The portion of the cervix protruding within the vagina is known as ectocervix. This is about 3 cm long. The rest of the cervix within the vaginal wall is labeled as endocervix. The ectocervix opens into vagina through a circular orifice known as external os. The passage between the external os and uterine cavity is known as endocervical canal. The opening of endocervical canal into the uterine cavity is known as internal os.

Histology of Cervix

Ectocervix

Ectocervix is lined by nonkeratinizing stratified squamous epithelium (**Fig. 7.2**). The epithelium can be divided into three parts: (1) Basal or parabasal zone—lowermost layer and contains basal and parabasal cells; (2) Intermediate zone—occupies the major part of the epithelium and is composed of intermediate cells; (3) Superficial zone—is the most differentiated superficial part of the epithelium and is composed of flattened superficial cells.

Endocervix

Mucosal lining of the endocervix is composed of a single layer of mucus-secreting columnar lining epithelium (**Fig. 7.3**). These cells are tall columnar with basally-placed nuclei giving rise to picket fence-like appearance.

Cervical stroma is composed of predominantly fibrous muscle and elastic tissue. There are many endocervical mucus secreting glands in the endocervical submucosal tissue.

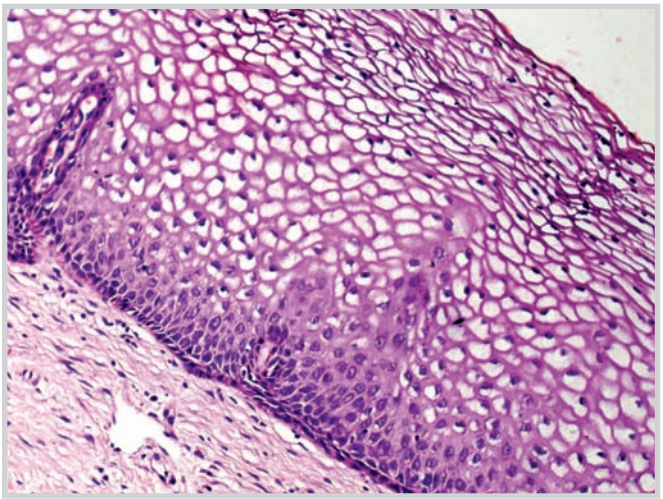


Fig. 7.2: Histology section of the normal ectocervical squamous epithelial lining (Hematoxylin and Eosin stain X HP)

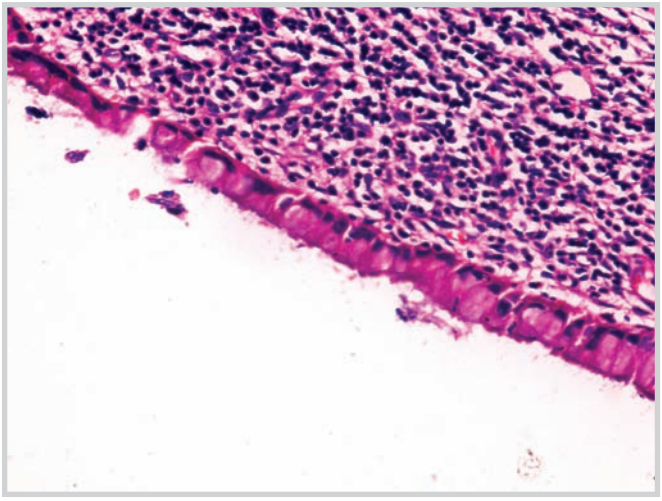


Fig. 7.3: Histology section of the normal endocervical lining epithelium (Hematoxylin and Eosin stain X HP)

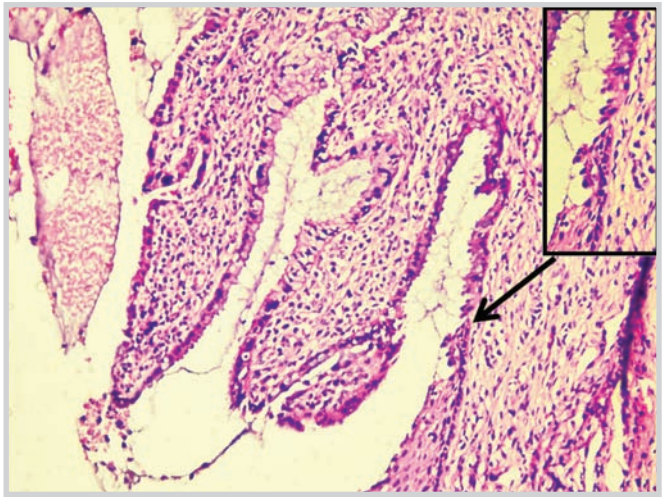


Fig. 7.4: Transformation zone or squamocolumnar junction of the cervix (Hematoxylin and Eosin stain X MP)

Transformation Zone

This is the junction between the stratified squamous epithelium and mucin-secreting columnar epithelium of the endocervix (**Fig. 7.4**). This is also known as squamocolumnar junction. The transformation zone is not a fixed area and depending on the situation it may shift toward ectocervix or endocervix (**Box 7.1**). Original transformation zone at the time of birth is toward the upper part of the cervix. At the time of child-bearing age or pregnancy the cervix enlarges and the transformation zone moves toward the external os. As the age increases, the squamocolumnar junction moves toward the upper part of the endocervical canal. In case of physiologic transformation zone, the endocervical epithelium undergoes squamous metaplasia and the new transformation zone is formed by metaplastic squamous epithelium and columnar epithelium.

The concept of the transformation zone is important because all the cervical squamous cell carcinoma originates from the new transformation zone and also the cervical preneoplastic conditions develop from this area. Therefore, sampling from transformation zone is important during cervical cancer screening.

BOX 7.1 **Transformation zone**

- Junction between the stratified squamous epithelium and columnar epithelium of the endocervix
- Cervical preneoplastic conditions and squamous cell carcinoma originates from the transformation zone
- Sampling target

Original transformation zone: The junction between the stratified squamous epithelium and mucin-secreting columnar epithelium of the endocervix

Physiologic transformation zone: The junction between the metaplastic squamous epithelium and columnar epithelium

Ovaries

These are two ovoid shaped, 3–5 cm diameter structures situated lateral to the fallopian tubes and uterus. Each ovary is attached to the uterus at its medial pole by utero-ovarian ligament. The ovary is divided into highly cellular outer cortex and less cellular, richly vascular inner medulla.

Surface Epithelium

Surface of the ovary is covered by single layer of modified mesothelial cells. The individual cells are cuboidal to columnar in appearance.

Stroma

Ovarian stroma is composed of spindle-shaped stromal cells along with multiple ovarian follicles in various stages of maturation.

Fallopian Tubes

Fallopian tubes extend from the cornual ends of the uterus laterally up to each ovary. It is of 9–11 cm in length. Tube at the ovarian end opens directly to the peritoneal cavity and is composed of 25 irregular fingers like extensions.

Histology

The fallopian tube has three layers: mucosal membrane, muscular layer and outer serosal layer. Mucous membrane consists of three types of columnar cells: (1) ciliated, (2) nonciliated secretory and (3) intercalary. There is no lamina propria and the mucosa is directly in contact with the muscle layer.

The muscle layer is composed of outer longitudinal and inner circular layer. The outermost serosa is lined by flattened mesothelial cells.

NORMAL CELLS IN CERVICAL SMEAR

The various normal cells are seen in routine cervical cytology smear on Papanicolaou's stained smear. It is necessary to identify these cells. **Table 7.1** highlights the differentiating features of various types of squamous cells on cervical cytology.

The ectocervix is lined by nonkeratinizing stratified squamous cells. The scraping of ectocervix therefore shows predominantly superficial squamous cells and intermediate cells. Occasionally, parabasal cells are also noted on the smear.

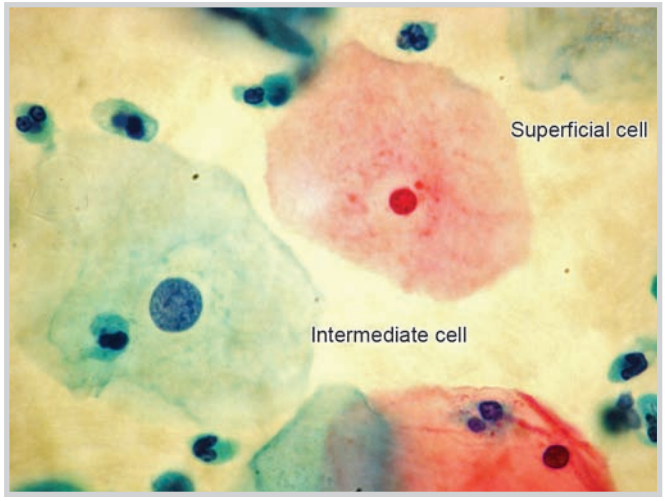


Fig. 7.5: Cytology smear showing superficial cell with abundant orangeophilic cytoplasm and central pyknotic nucleus. Smear also shows intermediate cell with abundant cytoplasm and vesicular nucleus (Papanicolaou's stain X HP).

Superficial Squamous Cells

These are large polyhedral cells with abundant thin pink to green cytoplasm and centrally placed small pyknotic nuclei. The diameter of a superficial cell is usually 30–45 microns and nucleus is about 5–7 microns (**Fig. 7.5**). The cells often show orangeophilic cytoplasm indicating intracellular keratin.

Intermediate Cells

These are large polyhedral cells with abundant pale green cytoplasm and centrally placed vesicular nucleus. The diameter of intermediate cell is usually 35–45 micron and nucleus is about 8–10 microns. Nucleus is the main differentiating point between superficial and intermediate cell (**Fig. 7.5**). The nucleus of the intermediate cell is vesicular with fine granular chromatin. Nuclear groove may be seen in intermediate cells as deep longitudinal fold. Intermediate cells with peripheral boat-like folding are known as navicular cells. This is due to progesterone effect on cervical epithelium. Navicular cells are seen in pregnant patients.

Parabasal and Basal Cells

These are small round to oval cells with dense cytoplasm and relatively large nuclei. Diameter of parabasal cells is 10–25 microns and nucleus is 6–8 micron, round with condensed chromatin (**Fig. 7.6**). Basal cells have almost same appearance except they are smaller in size. Parabasal cells are frequently seen in atrophic smear of postmenopausal patient.

Endocervical Cells

These cells are small round to columnar shaped with vacuolated cytoplasm and basally-placed nuclei (**Box 7.2**). The diameter of an endocervical cell is about 18–20 microns and nucleus is 8–10 microns. The nucleus is vesicular with finely granular chromatin having occasionally small nucleoli (**Figs 7.7A to C**). These cells are usually present in tight honeycomb-shaped clusters. On side view, the cells may give the appearance of picket fence. Endocervical cells may undergo tubal metaplasia (**Fig. 7.8**). The individual cells are columnar-shaped with cilia attached on terminal plate.

TABLE 7.1: Comparison of epithelial cells of cervical lining epithelium

Features	*Superficial*	*Intermediate*	*Parabasal*	*Endocervical*
Cell arrangement	Discrete	Discrete	Discrete	Honeycomb-shaped clusters
Cell size	Largest	Larger	Smaller	Smallest
Cell shape	Polyhedral	Polyhedral	Round	Round to columnar
Nucleus	Central pyknotic	Vesicular to fine granular chromatin	Round, occupies most of the cytoplasm; chromatin is fiinely granular	Round, central to eccentric
Cytoplasm	Orangeophilic	Pink to greenish cytoplasm	Dense cytoplasm	Vacuolated cytoplasm

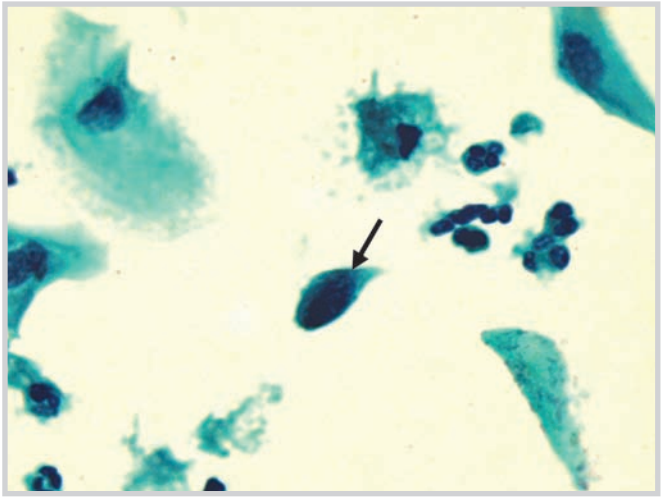


Fig. 7.6: Cytology smear showing parabasal cell with thick cytoplasm and centrally placed large nucleus (Papanicolaou's stain X HP)

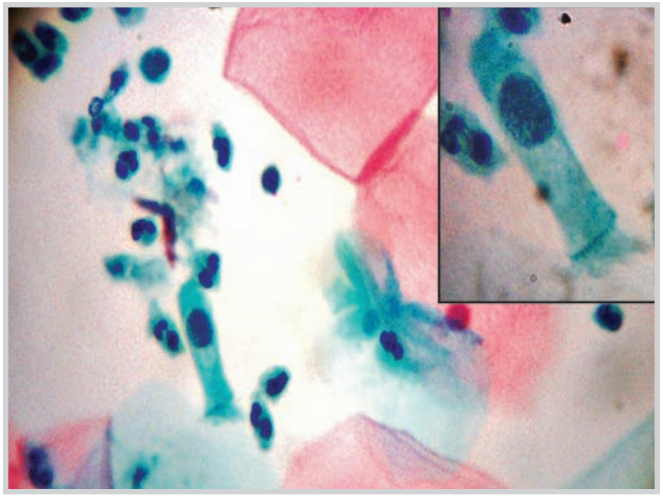


Fig. 7.8: Tubal metaplasia of the endocervical cells. Inset highlights endocervical cell with basal plate and cilia. (Papanicolaou stain X HP)

BOX 7.2 **Endocervical cells**

- Honeycomb-shaped clusters of cells
- On sideview, picket fence appearance at times
- Columnar-shaped cells with vacuolated cytoplasm and basally placed nuclei
- Vesicular nucleus with finely granular chromatin
- Small nucleoli
- Endocervical cells with tuft of cilia from terminal plate in tubal metaplasia

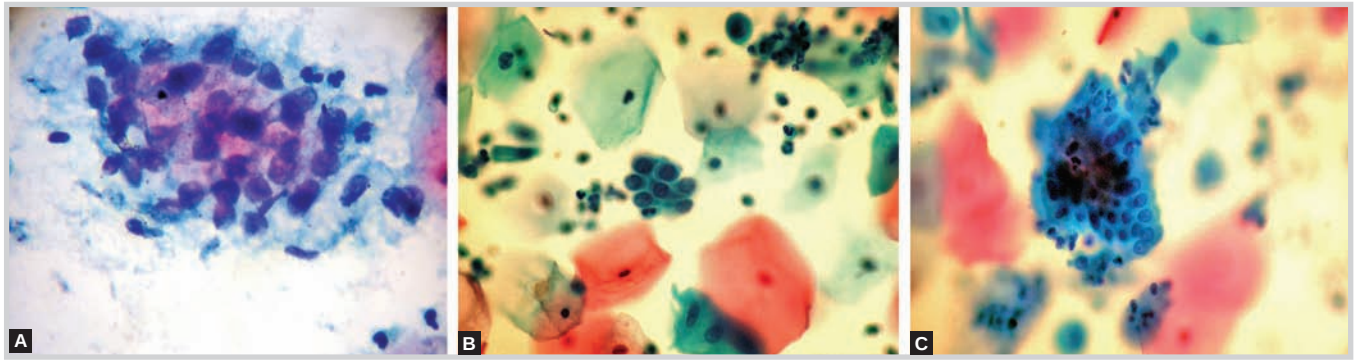
Metaplastic Squamous Cells

This is the most common change of endocervical cells. It usually occurs in transformation zone (**Box 7.3**). The glandular surface of the endocervix is replaced by squamous cell. The metaplastic cells are composed of immature parabasal cell. The cells are arranged in interlocking manner and one cell fits within another (**Fig. 7.9**). This gives a mosaic-like pattern. The cells of squamous metaplasia are relatively small with scanty cytoplasm. The nuclei are enlarged with slightly irregular contour and are mildly hyperchromatic.

Endometrial Cells

Endometrial cells may be spontaneously shed during the first half of menstrual cycle in the cervical smear (**Box 7.4**). Both glandular and superficial stromal cells may be present. Spontaneously, exfoliated normal-looking endometrial cells are also expected after birth of baby, abortion, endometrial polyp, endometritis and endometriosis. At times, clusters of endometrial cells may show dual population of cells with central small dark stromal cells and peripheral glandular cells with moderately vacuolated cytoplasm.

Endometrial cells are usually arranged in small tight spherical clusters (**Figs 7.10** and **7.11**). Individual cells are small round-shaped with round dark nuclei. The nuclei often show molding. Nucleoli are not seen in endometrial cells. Cytoplasm of the cells is scanty and occasionally may show vacuolations.



Figs 7.7A to C: (A) Endocervical cells in small cluster. Individual cells have moderate vacuolated cytoplasm and round monomorphic nuclei (Papanicolaou's stain X HP); (B) Small cluster of endocervical in flat sheet (Papanicolaou's stain X HP); (C) Large chunk of endocervical cells in SurePath liquid-based cytology smear (Papanicolaou's stain X HP)

BOX 7.3 **Immature squamous metaplasia**

- Single or sheets of cells
- Smaller parabasal type of cell
- Cytoplasm
  - Spider like cells with angulated projections
  - Cyanophilic
  - Dense
  - Sharp border
- Nucleus
  - Round to oval
  - Smooth outline
  - Fine chromatin
  - Small nucleoli
- High nucleocytoplasmic ratio

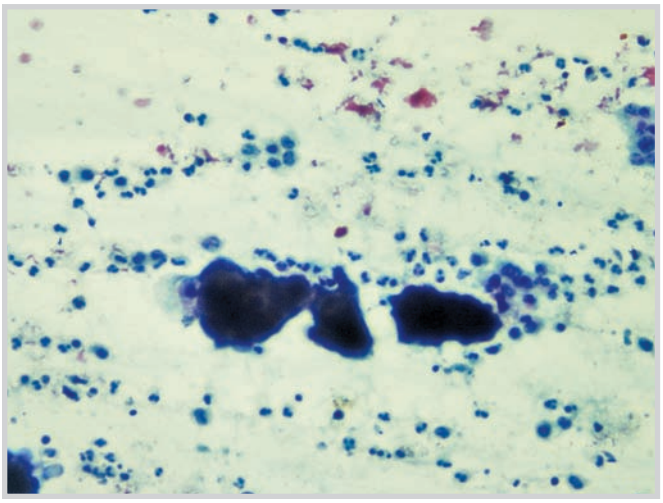


Fig. 7.10: Endometrial cells in small tight clusters (Papanicolaou's stain X MP)

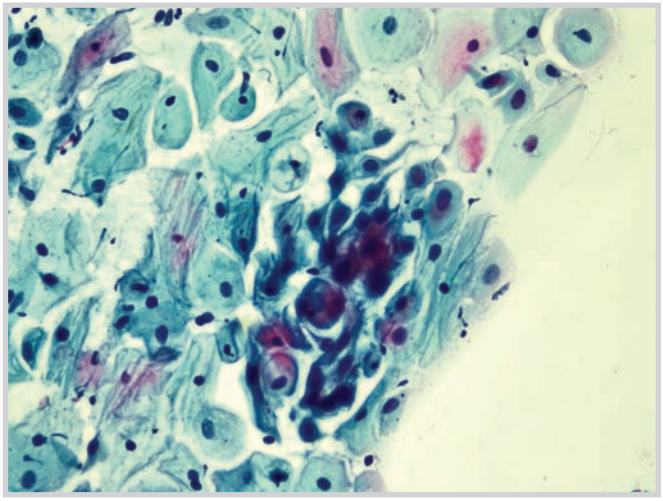


Fig. 7.9: Metaplastic endocervical cell in SurePath liquid-based cytology smear. Cells are arranged in interlocking manner and one cell fits within another (Papanicolaou's stain X HP)

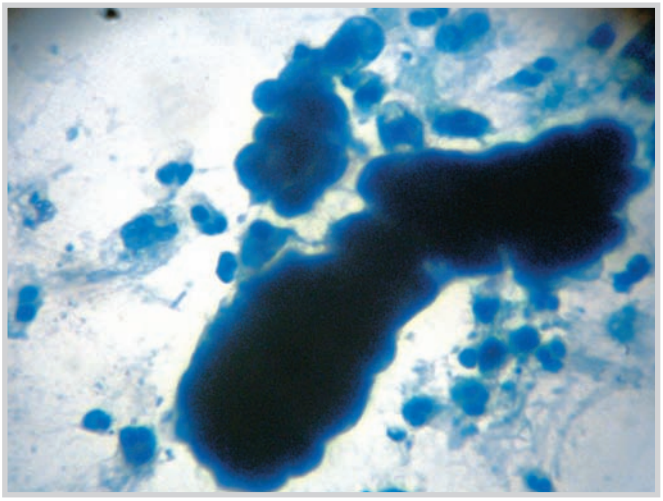


Fig. 7.11: High power magnification of endometrial cells in tight clusters. Individual cells have scanty cytoplasm and small hyperchromatic nucleus (Papanicolaou's stain X 420).

BOX 7.4 **Endometrial cells**

- Tight spherical clusters of cells
- Small round cells with scanty cytoplasm
- Cytoplasmic vacuolations
- Round dark nuclei with nuclear molding or angulated contour
- No nucleoli

Differential diagnosis: High grade squamous intraepithelial lesion, small cell nonkeratinizing squamous cell carcinoma and endometrial adenocarcinoma

Mimickers of Endometrial Cells

There are few mimickers of endometrial cells:

- High grade squamous intraepithelial lesion: The cells of high grade squamous intraepithelial lesions (HSILs) are usually bigger than endometrial cells. The nuclei are markedly hyperchromatic with irregular nuclear contour. Usually, these cells are arranged discretely or in loose clusters compared to tight spherical arrangement of endometrial cells.
- Poorly-differentiated squamous cell carcinoma: At times, poorly-differentiated squamous cells may pose diagnostic difficulties with endometrial cell clusters. Nuclear

hyperchromasia and pleomorphism are two helpful distinguishing features from endometrial cells.

- Well-differentiated adenocarcinoma: Occasionally, it is very difficult to distinguish well-differentiated adenocarcinoma of uterus from benign endometrial cells on cervical smears. In this condition, it is better to have complete investigations to exclude or confirm adenocarcinoma.

Inflammatory Cells

Various inflammatory cells such as polymorphonuclear leukocytes, lymphocytes and histiocytes may be present in the cervical smear.

Leukocytes

Polymorphonuclear leukocytes are often seen in cervical smears and their presence does not always indicate acute inflammation. Lymphocytes and plasma cells are noted in chronic inflammatory conditions.

Histiocytes (Macrophages)

These cells are often noted in cervical smears. The individual cells are large with centrally placed nuclei. The nuclear shape may be kidney-shaped. Cytoplasm often shows phagocytosed material. The presence of histiocytes in the cervical smear is a nonspecific finding and is noted in many conditions such as pregnancy, foreign body reactions, endometrial cancer, etc. Occasionally, multinucleated histiocytes are seen in foreign body granuloma, chronic inflammation or in postmenopausal atrophic smear (**Fig. 7.12**).

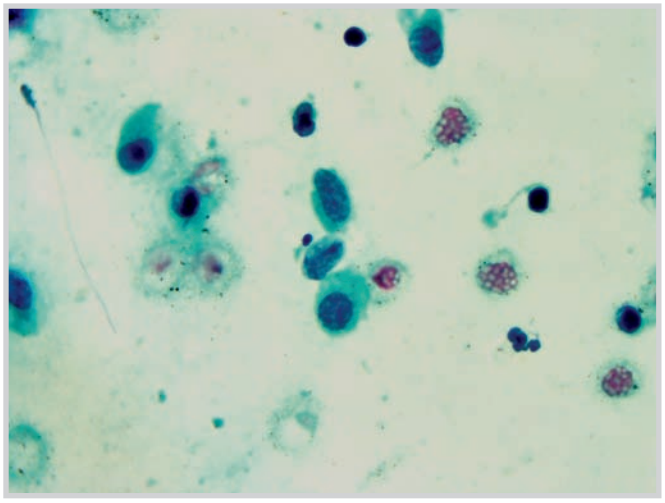


Fig. 7.12: Cluster of histiocytes and lymphocytes in cervical smear (Papanicolaou's stain X HP)

Trophoblastic and Decidual Cells

Rarely trophoblastic cells may be seen in cervical smear. The trophoblastic cell is large with abundant cytoplasm having multiple nuclei. Nuclei show mild irregular nuclear margin and fine granular chromatin (**Fig. 7.13**). Decidual cell is a large mononuclear cell with abundant cytoplasm. The nuclei are large vesicular with prominent nucleoli. In lack of history, it is difficult to identify the decidual cells with certainty and the cell may be confused with malignant cell.

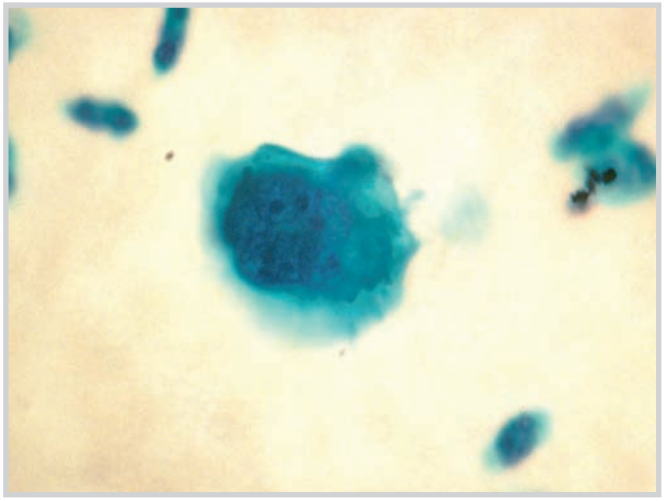


Fig. 7.13: Trophoblastic cell with large multiple nuclei (Papanicolaou's stain X HP)

OTHER CELLS

Spermatozoa

At times, sperms may be seen in cervical smear if the smear is taken sometime after sexual intercourse.

Lactobacilli

Lactobacilli are seen in cervical smear. These are Gram-positive rod-shaped bacilli normally present in the vaginal flora (**Fig. 7.14**). Lactobacilli metabolize the glycogen content of the exfoliated squamous cells and produce lactic acid. Lactic acid decreases pH of the vaginal tract and protects it from pathogenic invasion.

Ova of Parasites

Occasionally, ovum of parasites may be seen in cervical smears.

Cells of Adjacent Organ

Urothelial Cells

Rarely in case of uterovesical fistula, urothelial cells from urothelium of bladder may contaminate cervical smear. These cells may be difficult to identify.

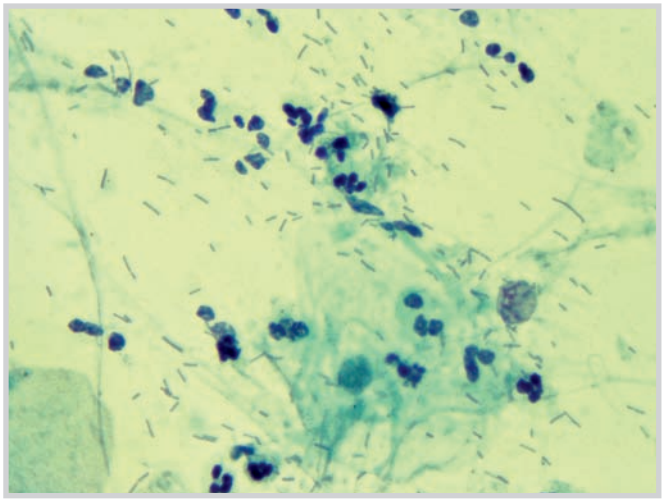


Fig. 7.14: Small scattered rod shaped *Lactobacilli* (Papanicolaou's stain X HP)

Colonic Cells

These are sheets of columnar epithelial cells and can be identified as rows of columnar cells similar to that of endocervical cells.

Acellular Material—Contaminants

Cervical smears may often show contaminants by dust of surgical gloves, lubricant jellies, etc.

Atrophic Smears

In case of atrophic smear, the predominant cell population is parabasal and basal cells (**Box 7.5**). There are minimal number of superficial squamous cells and intermediate cells. The parabasal cells are often present in sheets with crowded appearance (**Fig. 7.15**). There may be drying artifact and uniform nuclear enlargement. Atrophic epithelium is vulnerable to injury and inflammation. Therefore, the cells may show eosinophilic cytoplasm and nuclear changes of inflammation such as nuclear pyknosis and karyorrhexis. This should not be taken as significant. The smear may often show dark blue round-shaped amorphous material known as "blue blobs". These are most likely mucous globules or degenerated cells. In addition, the atrophic smear often contains elongated spindle-shaped cells that are probably artifact. These cells may be confused with spindle cell type of squamous cell carcinoma. In addition, blue blobs and background granular material may resemble necrosis and tumor diathesis and thereby may mislead the cytologists.

DIFFERENTIAL DIAGNOSIS

- High grade squamous intraepithelial lesions: Small cells with high N/C ratio of the cells in atrophic smear may be mistaken as HSIL. However, the cells in atrophic smears lack nuclear hyperchromasia and nuclear margin irregularity as noted in SIL.
- Invasive carcinoma: Atrophic smear may simulate tumor diathesis of invasive carcinoma due to fine granular dirty background and blue blobs. The individual cells do not show any cytological feature of malignancy.

BOX 7.5 **Atrophic smear**

- Predominant parabasal and basal cell population
- "Blue blobs": Dark blue round-shaped amorphous material
- Drying artifact with cytoplasmic eosinophilia and uniform nuclear enlargement
- Elongated spindle-shaped cells may be confused with malignancy
- Differential diagnosis HSIL, invasive carcinoma

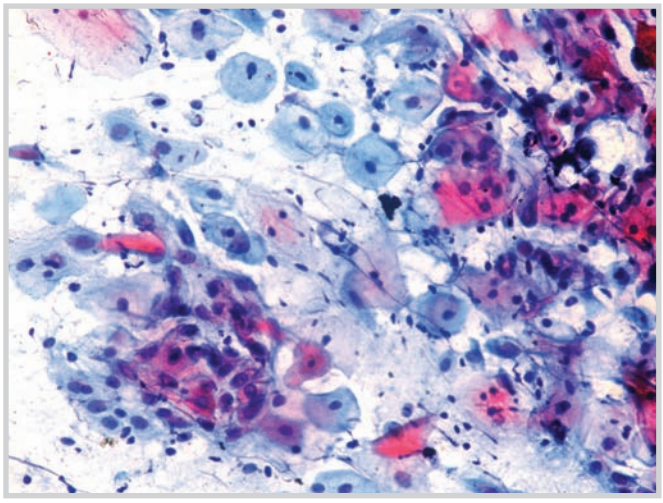


Fig. 7.15: Atrophic smear showing large number of parabasal cells (Papanicolaou's stain X HP)

BETHESDA SYSTEM OF REPORTING

In 1988 the National Cancer Institute, USA, sponsored a workshop in Bethesda, Maryland, among cytologists, cytotechnicians, pathologists and clinicians to develop a uniform terminology and reporting format of cervical cytology. The detailed format of the workshop was published later on as "The Bethesda System" (TBS) (**Box 7.6**).[1]

Afterward two other workshops were held in 1991 and 2001 subsequently, and the original framework and terminologies of TBS were modified.[2,3]

The Bethesda System 2001

The Bethesda System 2001 can be divided into three parts: specimen adequacy, general categorization and terminologies.

BOX 7.6 **The Bethesda system 2001**

Specimen type
Mention about conventional Papanicolaou smear or liquid-based cytology smear or other

Specimen adequacy
Satisfactory for evaluation:
- Adequate number of squamous cells:
  - Conventional smear: 8,000–12,000 squamous cells
  - Liquid-based cytology: 5,000 squamous cells
- Mention the presence or absence of endocervical cells or transformation zone components: at least 10 endocervical or metaplastic cells should be present, no need for cell clusters
- Any other quality elements such as drying artifact, obscured by blood or inflammation, etc. Partially obscured: 50–75% epithelial cells cannot be visualized, unsatisfactory: more than 75% epithelial cells cannot be visualized

Unsatisfactory for evaluation:
- Specimen rejected: mention the exact cause (such as broken slide)
- Specimen processed but unsatisfactory because: insufficient squamous cells or more than 75% of smear is obscured by blood or inflammatory cells

General categorization (optional)
- Negative for intraepithelial lesion or malignancy
- Epithelial cell abnormality
  - Interpretation/result
    - Organisms
      - *Trichomonas*
      - Bacterial vaginosis
      - Fungal morphology consistent with *Candida*
      - Bacterial morphology consistent with actinomycosis
      - Cellular morphology consistent with herpes simplex virus
    - Other non-neoplastic findings
      - Reactive cellular changes associated with
        - Inflammation
        - Intrauterine contraceptive device
        - Radiation
      - Glandular cell status post hysterectomy
      - Atrophy
- Others
  - Epithelial cell abnormality
    - Squamous cell
      - Atypical squamous cell
        - of undetermined significance (ASCUS)
        - cannot exclude HSIL (ASC-H)
      - Low-grade squamous intraepithelial lesion (LSIL)
      - High-grade squamous intraepithelial lesion (HSIL)
      - Squamous cell carcinoma
    - Glandular cell
      - Atypical glandular cells: Specify endocervical, endometrial or not otherwise specified
      - Atypical glandular cells favor neoplastic: Specify endocervical, endometrial or not otherwise specified
      - Endocervical adenocarcinoma in situ
      - Adenocarcinoma
  - Other
    - Endometrial cells in a woman more than 40 years of age

Automated Review and ancillary testing
Educational notes and suggestions

Specimen Adequacy

Originally in TBS 1988 and TBS 1991, there were three parts in specimen adequacy: "satisfactory", "satisfactory but limited" and "unsatisfactory". The Bethesda system 2001 maintains only "satisfactory for evaluation" and "unsatisfactory" categories and eliminates "satisfactory but limited" category. The "satisfactory but limited" category was considered confusing to many clinicians and prompted repeat. So the elimination of this category gives clear and reproducible indications about the adequacy of the sample. However, the option was kept about providing information on transformation zone to improve the quality of the sample collection.

Cellularity

Previously in TBS 1991 format, it was considered that at least 10% of the surface area of the slide should be covered by well-visualized and well-preserved squamous cells for the adequate amount of squamous cells. In contrast, TBS 2001 provided the quantitative numerical estimate for the adequacy of squamous cells.

Adequate Number of Cells

Conventional smear: 8,000–12,000 squamous cells.
Liquid-based cytology: 5,000 squamous cells.

Only the well-visualized and well-preserved squamous cells should be taken into consideration. The approximate number of cells should be estimated only and the cytologists are not expected to count individual cells. The Bethesda System 2001 published the reference images on their website to help in the estimation of the number of cells on conventional smear and liquid-based preparations.[4]

Liquid based cytology (LBC) preparation requires less number of squamous cells to fulfil the adequacy criteria because LBC technique represents homogenous population of cells. A minimum number of 5,000 cells for adequate LBC was kept on the basis of correlation between cellularity and number of false negative cases.[5]

ENDOCERVICAL CELLS

In TBS 2001, the mention of the presence of endocervical cell or transformation zone component is needed. There should be at least 10 well-preserved endocervical cells or metaplastic cells, however, the clusters of cells are not required.

The presence of endocervical cells and sampling adequacy is a bit contradictory. It was believed that for sampling adequacy the presence of endocervical cells are mandatory because squamous intraepithelial lesion (SIL) is more frequent when endocervical cells are present on the smear.[6] However, further studies did not show any association between false-negative cervical smear and the absence of endocervical cells.[7] Currently, the absence of endocervical cell is not considered as an unsatisfactory smear.

General Categorization

"General categorization" is kept an optional component in TBS 2001. The previous two categories "within normal limit" and "benign cellular changes" described in TBS 1991 have been clubbed together in a single category as "negative for intraepithelial lesion or malignancy" in TBS 2001. The category "other" has been kept for cases that do not show any morphological abnormality, however, the findings should be mentioned to avoid increased risk such as "the presence of benign looking endometrial cells in woman over 40 years of age".

These categories are mutually exclusive. However, if several findings are present in one smear then the specimen should be categorized according to the most clinically significant lesion (such as epithelial abnormality).

Interpretation and Result

The cervical smear report is primarily a screening test and the exact diagnosis of the patient depends on clinical history and other laboratory investigations. Therefore in the 2001 TBS, the term diagnosis has been replaced by interpretation or result.

Negative For Intraepithelial Lesion or Malignancy (NILM)

The specimen in absence of epithelial cell abnormality is reported as NILM. The term "infection" has been replaced by "organism" in TBS 2001 because the presence of some organisms may not indicate clinical infection.

There is optional choice to report other non-neoplastic findings such as changes due to reparative processes, radiation, atrophy, and intrauterine contraceptive devices. The cytological features of these lesions are important to recognize to avoid false positive cases because the cellular features of these cases may resemble intraepithelial lesions.[8] The presence of endometrial cell has a significantly low risk of endometrial cancer and also in absence of proper clinical history a meaningful interpretation of the presence of endometrial cell is difficult. Therefore, TBS 2001 introduced a new category "other" to report the benign appearing endometrial cells in woman over 40 years of age, which was kept previously in the category of epithelial cell abnormality in TBS 1991.

Epithelial Cell Abnormalities

Atypical Squamous Cells

Previously in TBS, the term atypical squamous cell of undetermined significance (ASCUS) was used to designate the cellular changes which were more than reactive changes but quantitatively or qualitatively fell short of a definitive diagnosis of SIL. The cytopathologists were encouraged to label ASCUS as "not otherwise specified", "favor reactive" and "favor SIL". It was noted that large proportion of ASCUS was reported as ASCUS "not otherwise specified". In 2001 TBS, "atypical squamous cell" is qualified as "atypical squamous cell of undetermined significance (ASCUS)" or "atypical squamous cells, cannot exclude HSIL" (ASC-H). The category ASC, favoring reactive atypia, is eliminated and the cytologists have to take decision judiciously to put the cases either as NILM or in ASC.

Atypical squamous cells, cannot exclude HSIL suggests that cytological features are close to HSIL but do not fully support the

definitive interpretation of HSIL. According to TBS 2001, ASC-H includes HSIL and its mimics and will include 5–10% of ASC cases overall.

Squamous Intraepithelial Lesion

The Bethesda System 2001 workshop kept two tiered LSIL/HSIL terminology as was suggested by previous TBS. The dichotomous division of SIL is kept because it was thought that LSIL and HSIL are clinically different. LSIL is a transient infection of HPV and rarely progress to higher grade, whereas HSIL is due to persistent infection and has higher risk of progression.[9] The other supporting evidences of this two-tier system are: (1) The two tier system in TBS attempts to separate low risk LSIL from the high risk HSIL on the basis of morphology, (2) LSIL versus HSIL is reproducible, and (3) Subdividing HSIL into CIN 2 and CIN3 may not be reproducible.

High Grade Squamous Intraepithelial Lesion Cannot Exclude Invasion

In certain situations, it may be difficult to distinguish HSIL form an invasive carcinoma on the basis of cytological features. In this situation, cytologists may apply this terminology to increase clinician's concern on that particular case.

Squamous Cell Carcinoma

It represents the invasive squamous cell carcinoma. Usually tumor diathesis is present in invasive squamous cell carcinoma. In TBS, there is no option to differentiate keratinizing from non-keratinizing squamous cell carcinoma in cytology smear.

Atypical Glandular Lesion

This terminology is applied to those cases where the cytological features are more than reactive glandular changes but fall short to have a definitive diagnosis of adenocarcinoma. The terminology of atypical glandular cells has been significantly changed in TBS 2001 and the term "atypical glandular cells of undetermined significance (AGUS)" has been eliminated to avoid confusion with ASC-US. The glandular cell abnormalities have been classified as "atypical endocervical, endometrial or glandular cell". The pathologists are encouraged to identify the origin of the atypical glandular cells (whether endocervical or endometrial) and specify in report, if not possible, then they may just mention "atypical glandular cell". The term "atypical glandular cell-favors reactive" has been eliminated in TBS 2001, because it was considered as misleading.

Automated Review and Ancillary Testing

If the slides are scanned in automated scanning system then in the cytology report there should be a complete mention of the name of the instrumentation and the results of the automated system. Similarly, if any other ancillary test is done such as screening of high risk HPV test then the result should be mentioned concurrently or as an addendum with cytology report.

Educational Notes and Suggestions

The cytologists may write brief educational notes or suggestions in the report. The comments and suggestions should be according to guidelines of the professional societies. It is preferable not to communicate with the patient directly as this may interfere the patient-clinician relationship.

ORGANISMS AND INFECTION

Inflammatory Smear

Acute Cervicitis

In case of acute inflammation, the smear shows heavy inflammatory exudates predominantly composed of polymorphonuclear leukocytes, necrotic debris and inflammatory changes in the epithelium (**Box 7.7**). The inflammatory cells are usually adhered to the epithelial cells (**Fig. 7.16**). Usually, the parabasal cells are increased in number and the epithelial cells often show eosinophilia. The individual cells also show degenerative changes such as cytoplasmic perinuclear vacuoles, nuclear pyknosis, karyorrhexis and karyolysis.

Bacterial Vaginosis

Bacterial vaginosis (BV) is a fairly common infection and affects large number of patients worldwide. The patients have thin milky vaginal discharge with foul fishy smell. Many of them may be asymptomatic also. Previously, it was believed that *Gardnerella vaginalis* is solely responsible for this infection. However, now it is evident that BV can be caused by infections with other bacteria in Clostridiales order. It is now suggested to report as "shift in bacterial flora" rather than to make the specific diagnosis of *G vaginalis* infection. BV cases are positive for Whiff's test. In this test, 10% potassium hydroxide is added on the slide of vaginal fluid. The production of fishy smell indicates a positive test.

Smear shows numerous small coccobacilli replacing the normal lactobacilli giving a filmy background (**Box 7.8**). There may be variable amount of acute inflammatory cells. Clue cells are one of the diagnostic features of BV (**Fig. 7.17**), however, they are not specific for BV. These are large polyhedral squamous cells having transparent cytoplasm filled up by multiple tiny coccobacilli.

BOX 7.7 Inflammation

- Polymorphonuclear leukocytes infiltration
- Necrotic debris
- Inflammatory cells stick to the epithelial cells
- Increased number of parabasal cells
- Eosinophilia of the cell cytoplasm
- Degenerative changes
  - Cytoplasm: Vacuoles
  - Nucleus: Pyknosis, karyorrhexis, karyolysis

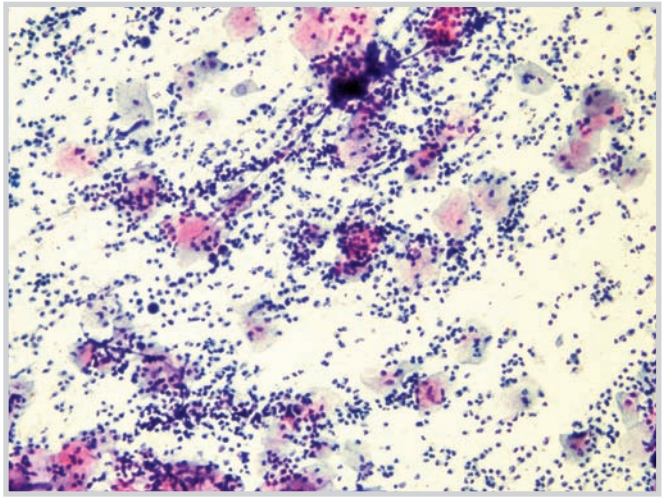


Fig. 7.16: Inflammatory cervical smear shows polymorphs adhered with epithelial cells (Papanicolaou's stain X MP)

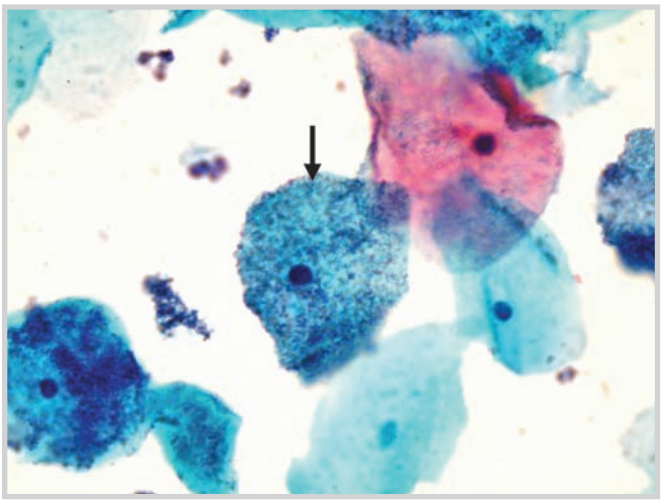


Fig. 7.17: Classical clue cells with multiple coccobacilli in the cytoplasm in bacterial vaginosis infection (Papanicolaou's stain X HP)

BOX 7.8 **Bacterial vaginosis (shift in bacterial flora)**

- The patient has thin milky vaginal discharge with foul fishy smell
- Caused by *Gardnerella vaginalis* and other bacteria
- Positive for Whiff's test: Adding 10 % potassium hydroxide on vaginal fluid produces fishy smell

Cytology smear

- Filmy background
- Absence of lactobacilli
- Numerous small coccobacilli and curved bacilli and
- Clue cells: Polygonal squamous cells filled with small coccobacilli

BOX 7.9 ***Trichomonas vaginalis* (TV)**

- Burning sensation, severe itching, and foul smelling vaginal discharge

Morphology of TV

- 15 to 30 microns long
- Round-to-oval "pear-shaped" organisms
- Indistinct vesicular nucleus: important feature
- Red cytoplasmic granules

Background

- Dirty, fuzzy
- "Cannon ball" or "pus ball": aggregates of neutrophils around the surface of the squamous cells
- Associated with *Leptothrix* (80% cases)

Differential diagnosis: Cytoplasmic fragments of epithelial cells

Trichomonas vaginalis

Trichomonas vaginalis (TV) is the most common infection in the female genital tract (**Box 7.9**). The patient usually complains of burning sensation, severe itching, and foul smelling vaginal discharge. In addition, the patient may have lower urinary tract infection.[8] Considerable number of patients (up to 50%) may be asymptomatic.

Trichomonas vaginalis is well visualized in Papanicolaou's stained cervical smear as 15–30 microns long, round-to-oval "pear-shaped" organisms with an indistinct eccentric vesicular nucleus and red cytoplasmic granules (**Fig. 7.18**). Flagella may be seen in LBC preparation. In majority of the cases, TV is associated with long branching curved bacilli known as *Leptothrix*. The background of smear is usually dirty and often shows aggregates of neutrophils around the surface of the squamous cells known as "cannon ball" or "pus ball". In addition, squamous cells show perinuclear halo and increased eosinophilia.[10]

Candida

Candida albicans and *Candida glabrata* are two common Candidal species that infect lower female genital tract (**Box 7.10**). The patient complains of burning, itching and thick white discharge. However, a good number of patients may be asymptomatic.

Cytomorphology

Candida may be in two forms: conidia (yeast) form and fungus (pseudohyphae) form. The yeast forms are pink colored, oval 3–7 microns diameter encapsulated regular structure. The fungus forms are long slender non-septate structures which are known as pseudohyphae (**Fig. 7.19**).

The associated squamous cells show nuclear enlargement, perinuclear haloes and increased eosinophilia. In addition, background shows many fragmented neutrophils.

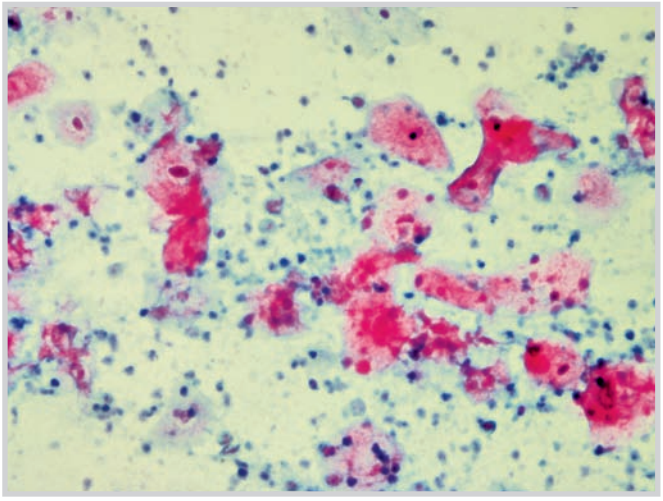


Fig. 7.18: Round shaped *Trichomonas vaginalis* in cervical smear (Papanicolaou's stain X HP)

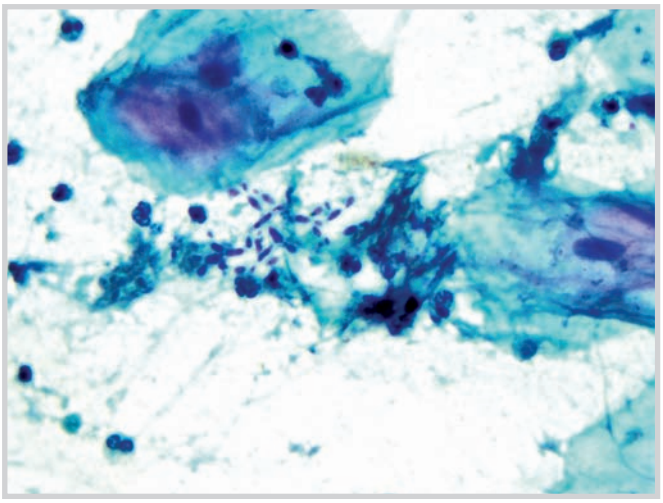


Fig. 7.19: Candidal spore and pseudohyphae in cervical smear (Papanicolaou's stain X HP)

BOX 7.10 Candida

- Two common candidal infection: *Candida albicans* and *Candida glabrata*
- Complaints: Burning, itching and thick white discharge
- Conidia (yeast) form: Pink colored, oval 3–7 microns diameter encapsulated regular structure
- Fungus (pseudohyphae) form: Long slender non-septate structure

Other changes:

- Squamous cells: Nuclear enlargement, perinuclear haloes and increased eosinophilia
- Background: Many fragmented neutrophils

Comments:

- Perinuclear halo in the squamous cells may often be mistaken as koilocyosis
- Nuclear atypia of the squamous cell may simulate atypical squamous cell

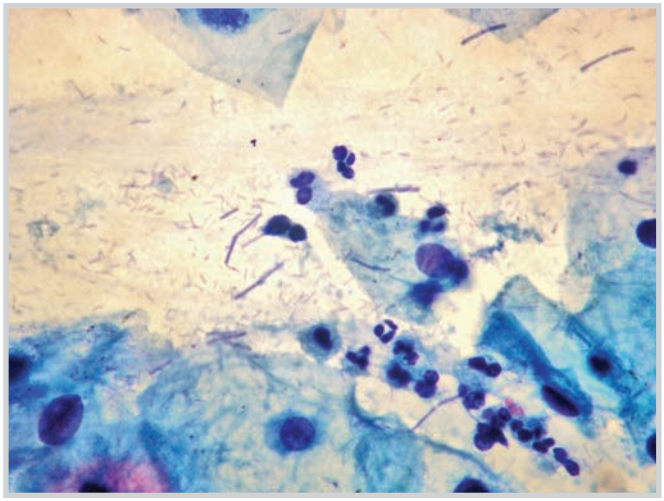


Fig. 7.20: Long slender *Leptothrix* in cervical smear (Papanicolaou's stain X HP)

Differential Diagnosis

1. Thin mucus strands may often be mistaken as candidal pseudohyphae. However, they are pale blue in color.
2. Perinuclear halo in the squamous cells may often be mistaken as koilocyosis.
3. Nuclear atypia of the squamous cell may simulate atypical squamous cell.

Leptothrix

It is a type of lactobacillus and is a Gram-negative, non spore forming anaerobic organism. It is a thin, segmented, long filamentous organism (**Fig. 7.20**). *Leptothrix* is commonly associated with *Trichomonas* infection.

Actinomycosis

Actinomycosis (**Box 7.11**) are Gram-positive aerobic organisms commonly associated with intrauterine contraceptive devices (IUCD). The patients are asymptomatic or may show foul smelling vaginal discharge.

Cytomorphology

The clumped of grayish blue filamentous bacteria radiate from a dense center. The organisms are thick and beaded in appearance. Overall they give a cotton ball appearance (**Fig. 7.21**). Background of the smear shows numerous polymorphonuclear leukocytes, macrophages and occasional multinucleated giant cells.

Cytomegalovirus Infection

Cytomegalovirus is a DNA virus of the Herpes family (**Box 7.12**). It may be noted both in immunocompetent and immunocompromised patients. Morphologically, the infected cells are mononuclear and markedly enlarged with a prominent basophilic intranuclear inclusion. Cytoplasm also shows multiple granular small inclusions.

Herpes Simplex

Herpes simplex type II (**Box 7.13**) causes genital infection. This is a sexually transmitted infection. Clinically, there are multiple small vesicles filled with clear fluid. Pustules may appear. Within 10–15 days the ulcerative lesions heal and re-epithelialization occurs.

Cytomorphology

Infected cells are large, multinucleated with molding of the nucleus. Homogenization of nuclear content gives rise to ground glass appearance. Large eosinophilic intranuclear inclusion with surrounding clear area is noted. Cytoplasm of the infected cell is dense and opaque.

BOX 7.11 Actinomycosis

- Gram-positive aerobic organism
- Commonly associated with intrauterine contraceptive devices (IUCD)
- Asymptomatic or may show foul smelling vaginal discharge containing Sulphur granules
- Clumped of grayish blue filamentous bacteria radiate from a dense center.
- Cotton ball appearance
- Background : Numerous polymorphonuclear leukocytes, macrophages and occasional multinucleated giant cells

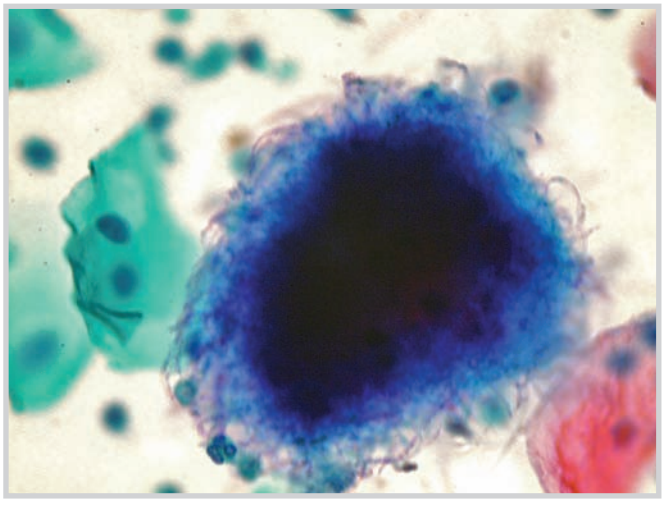


Fig. 7.21: *Actinomycosis* of cervical smear. The filamentous bacteria radiates from a dense center (Papanicolaou's stain X HP)

BOX 7.12 Cytomegalovirus

- DNA virus of the herpes family
- Mononuclear cells
- Marked cytomegaly and nucleomegaly
- Prominent basophilic intranuclear inclusion
- Multiple granular small inclusions in cytoplasm

BOX 7.13 Herpes simplex

- Sexually transmitted infection
- Infected cell are large
- Multinucleated with molding of the nucleus
- Ground glass appearance
- Large eosinophilic intranuclear inclusion
- Bizarre nucleus may be noted

Chlamydia trachomatis

Chlamydia trachomatis (**Box 7.14**) is the most common sexually transmitted infection. It is a Gram-negative organism. Most of the patients are asymptomatic. The symptomatic patients may have urethritis, vaginitis, cervicitis and pelvic inflammatory disease.

Initially, tiny coccoid bodies surrounded by narrow clear zones are noted in the cytoplasm of squamous and endocervical cells. The infected cells in course of time may show the moth eaten appearance of the cytoplasm. Later on, clearly visible central acidophilic or basophilic inclusion bodies are seen in the cytoplasm (**Fig. 7.22**). *Chlamydia trachomatis* infection is associated with heavy infiltration by acute inflammatory cells. This may obscure the cytoplasmic detail. Therefore, it is difficult to distinguish degenerative changes due to other causes and infection by *Chlamydia trachomatis*. The diagnosis of *Chlamydia trachomatis* should always be confirmed by culture.

CYTOLOGICAL FEATURES OF REPARATIVE AND REGENERATIVE CHANGES

In case of injury or chronic irritation, the reserve cells of the cervical lining epithelium proliferate and replenish the loss. Injury of the lining epithelium of the cervix may be due to various causes such as recent hysterectomy, severe cervicitis, history of conization of cervix, etc. (**Box 7.15**).

Cytology

The cells are often arranged as two-dimensional flat cohesive sheets. Individual cells are arranged in one direction giving the appearance of "school of fish". The cells are enlarged with well-defined cytoplasmic margin. Nuclei are enlarged and are pleomorphic. Nuclear chromatin is fine with single to multiple prominent nucleoli. Mitotic activity may be seen. In addition, background may show inflammatory exudates and blood.

BOX 7.14 ***Chlamydia trachomatis***

- The most common sexually transmitted infection
- Tiny coccoid bodies surrounded by narrow clear zones are noted in the cytoplasm of squamous and endocervical cells
- Moth eaten appearance of the cytoplasm
- Central acidophilic or basophilic inclusion bodies in the cytoplasm
- Culture is mandatory for confirmation

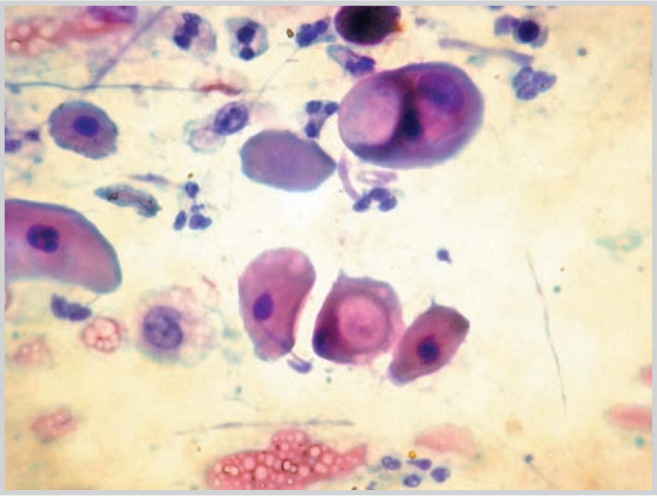


Fig. 7.22: Intracytoplasmic inclusion in case of *Chlamydia trachomatis* infection (Papanicolaou's stain X HP)

BOX 7.15 **Repair**

- Occurs after injury or chronic irritation
- Two-dimensional flat cohesive sheets
- Individual cell are arranged in one direction: "school of fish appearance"
- Nucleus
  - Enlarged
  - Pleomorphic
  - Smooth nuclear border
  - Hyperchromatic
  - Single to multiple prominent nucleoli
- Cytoplasm: Abundant may be vacuolated
- Low N/C ratio
- Neutrophils are often seen
- Mitotic activity may be seen
- Mimics malignancy

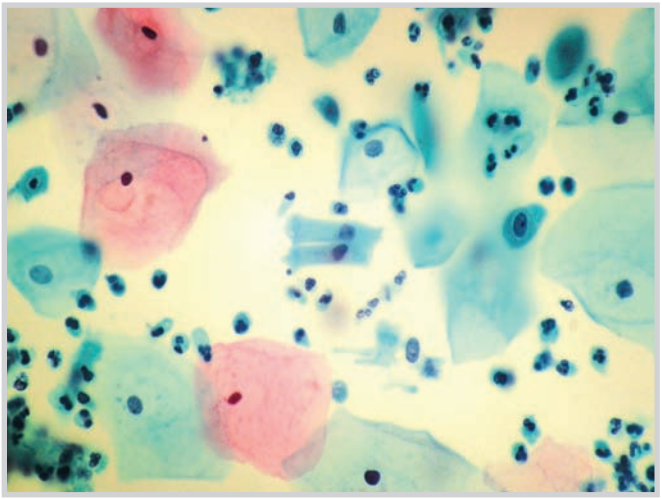


Fig. 7.23: Cervical smear showing tubal metaplasia in LBC (SurePath) preparation. Endocervical cells with terminal plate and cilia (Papanicolaou's stain X HP)

Differential Diagnosis

Carcinoma

The cytological features of repair may simulate carcinoma and therefore, one should be careful in interpretation of this type of smear. The cells in repair are almost always cohesive and single discrete pleomorphic cells are rarely seen.

Parakeratosis

At times, the superficial layer of squamous cells of cervix may show small nucleated squamous cells. This is caused by chronic irritation. The cells are arranged in isolated or loose sheets. Individual cells are small, polygonal with eosinophilic to orangeophilic cytoplasm. Nuclei are small, uniform and pyknotic. The persistence presence of these cells in cervical smear needs close follow-up.

Tubal Metaplasia

Occasionally, due to chronic irritation the cervical lining, epithelium undergoes tubal metaplasia. Cervical smear normally may show small number of ciliated columnar cells. However, in case of tubal metaplasia, the number of such cells is more. The cells are arranged in small groups or in pseudostratified strips. Individual cells are columnar in appearance with discrete vacuoles. The one end of the cell has a terminal bar and cilia arise from the terminal bar (**Fig. 7.23**). Nuclei of the cells are centrally located, round to oval, with mildly enlarged and atypical. Occasionally, tubal metaplasia may shed abnormal cells that may be mistaken for malignancy.[11]

Intrauterine Contraceptive Devices-related Changes

In case of IUCD use (**Box 7.16**), both endocervical and endometrial cells show abnormality. Tail and body of IUCD cause irritation of adjacent endocervical epithelium and endometrium. As mentioned before, IUCD is often associated with actinomycosis infection.

BOX 7.16 IUCD-related changes

- Associated with actinomycosis infection (25%).
- Two distinct types of cells:
  - "Bubble gum cell":
    - Cells with large vacuolated cytoplasm
    - Enlarged nucleus having prominent nucleoli
  - IUCD cells
    - Small round to oval cell with high nucleocytoplasmic ratio
    - Hyperchromatic nucleus
- Background: Psammoma bodies or calcification may be seen

Mimicker: Adenocarcinoma: Features such as absence of tumor diathesis, degenerative changes, background inflammation are common in IUD smears.

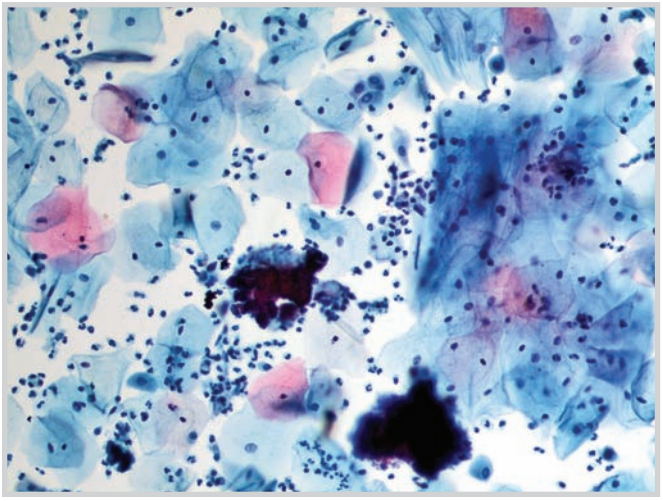


Fig. 7.24: Dense calcified material in case of IUCD use in liquid-based cytology preparation (SurePath) (Papanicolaou's stain X HP)

Two distinct types of cells may be noted in IUCD users.[12] One type of cell is glandular and is arranged singly or in small clusters. Individual cells have vacuolated cytoplasm with enlarged nucleus having prominent nucleoli. These cells with large vacuolated cytoplasm are often referred as "bubble gum cell". Occasionally, psammoma bodies or calcification may be seen (**Fig. 7.24**). The other cell type is small round to oval with high nucleocytoplasmic ratio and hyperchromatic nucleus. These cells are probably of endometrial in origin and are known as "IUCD" cells.

The cytological features of IUCD users may often simulate adenocarcinoma of endocervix or endometrium and should be carefully interpreted. Clinical history of IUCD use may be helpful in this situation.

BOX 7.17 Radiation effect

Acute effect:
- Cells: markedly enlarged
- Cytoplasm: abundant and vacuolated
- Multinucleation is frequent
- Nucleus: enlarged mildly pleomorphic
- Nuclear-cytoplasmic ratio low
- Nuclear chromatin : Smudged
- Nucleoli: Single to multiple prominent

Chronic effect
- Cell: Mildly enlarged
- Sheets of squamous cell with spindle-shaped elongated nuclei resembling smooth muscle cell

Radiation-induced Changes

Radiation-induced changes in cervix may be acute or chronic. The effects of radiotherapy are on benign epithelial cells and on cancer cells (**Box 7.17**).

Changes in Acute Radiation Effect

The cells are enlarged with marked nucleomegaly. The cytoplasm is abundant and vacuolated. The nuclear chromatin is smudged with single to multiple prominent nucleoli (**Fig. 7.25**). The squamous cells may show multinucleation, bizarre form and hyperchromasia. These acute changes in the squamous epithelium regress in course of time.

Chronic Radiation Effect

The cells show mild nuclear enlargement. However, no cytoplasmic vacuolations and nuclear changes are seen. The number of multinucleated cell reduces. There may be sheets of squamous cell with spindle-shaped elongated nuclei resembling smooth muscle cell (**Fig. 7.26**).

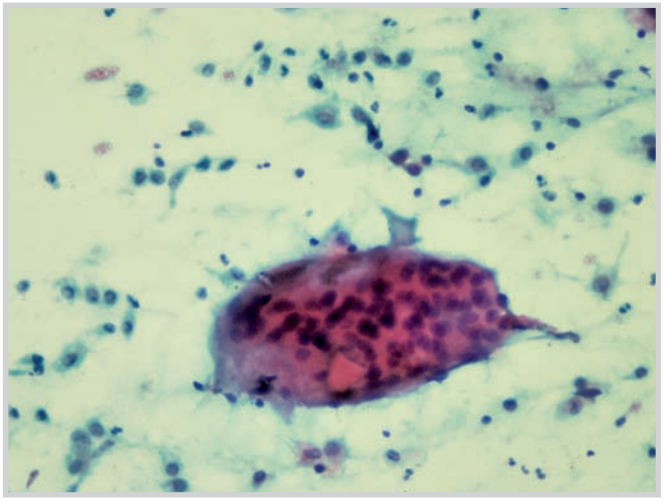


Fig. 7.25: Radiation changes in cervical smear showing multinucleated cell (Papanicolaou's stain X MP)

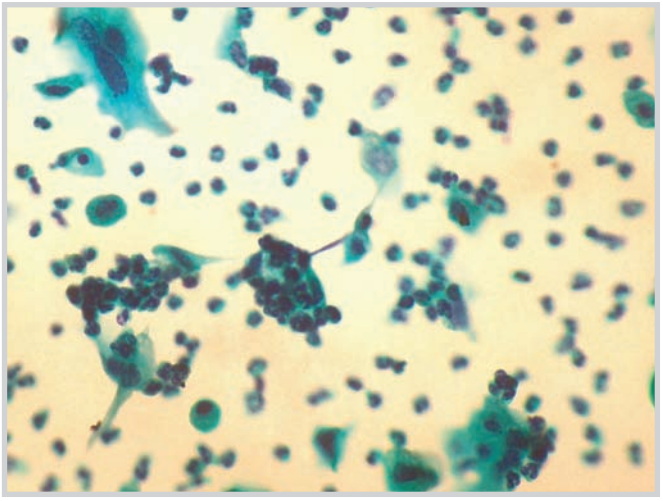


Fig. 7.26: Radiation changes in cervical smear in liquid-based cytology preparation (Papanicolaou's stain X HP)

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CHAPTER 8

Cervical Carcinogenesis, Preneoplastic and Neoplastic Condition

Chapter Contents

HUMAN PAPILLOMA VIRUS AND CERVICAL CARCINOGENESIS

Human papilloma virus (HPV) infection is the major cause of cervical carcinogenesis. It is a sexually transmitted infection occurring in both men and women. HPV infection is very common and most infected individuals eliminate HPV without any recognizable evidence of disease. Occasionally, HPV-infected individuals progresses to cervical carcinoma. However, it has been shown that all cases of cervical carcinoma is positive for HPV DNA.

Prevalence of HPV

It is estimated that HPV prevalence in world's woman population is about 2–44%.[1] This wide variation of range may be due to difference of sensitivity of DNA assay and age range of the population studied.

Different Subtypes of HPV

More than 100 different subtypes of HPV are known and only minorities of them are involved in cervical carcinogenesis. About 30 subtypes of HPV-infected genital tract and only minority of them cause cervical cancer. HPV subtypes can be divided into low-risk and high-risk depending on their oncogenic potential. Low-risk HPV types are 6, 11, 42–44, 54, 61, 70, 72 and 81 and high-risk HPV types are 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 61, 66, 68, 73, and 82 (**Table 8.1**). Low-risk HPV subtypes are usually associated with condyloma warts. High-risk subtypes are associated with squamous intraepithelial lesions and are at higher risk for progression of malignancy.

Risk Factors Associated with Cervical Cancer

In addition to HPV infection, other cofactors are also required for cervical carcinogenesis, such as smoking, nutritional deficiency, high parity, absence of male circumcision, long-term oral contraceptive pill (OCP) use, immune suppression and co-infection with human, immunodeficiency virus, herpes simplex virus and *Chlamydia trachomatis*.

Natural Course of Infection

The most HPV infections occur soon after initiation of the sexual activity and the infections are transient. Prevalence of

TABLE 8.1: Types of human papilloma virus (HPV)

Risk classification	*HPV Types*	*Lesions*
Low risk	6, 11, 42, 43, 44, 54, 61, 70, 72, 81	Condyloma, LSIL
High risk	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 61, 66, 68, 73, 82	LSIL, HSIL, carcinoma

Abbreviations: HSIL—high grade intraepithelial lesion; LSIL—low grade squamous intraepithelial lesion

HPV is highest at 18–25 years of age, and then declines as the age increases. It has been shown that the majority of women infected with a specific HPV type do not show evidence of that same type about one year later. The initial HPV infection clears of rapidly within 12–18 months.[2] Infection by multiple types of HPV is common and there is higher chance of persistence of HPV infection in this situation.[2] It was originally believed that there is a natural progression of cervical disease from cervical intraepithelial neoplasia 1 (CIN 1) to CIN 2 and CIN 2 to CIN 3 followed by invasive carcinoma.[3] However, it is now known that the majority of low-grade lesions regress without treatment and a significant proportion of high-grade lesions progress to invasive cancer if left untreated. Up to 35% cases of low grade squamous intraepithelial lesions (LSIL) are associated with HPV 16 and 18 and 10% cases of LSIL are associated with HPV type 6 and 11 infection.[4] LSIL associated with HPV 16 and 18 are more frequently progress to higher grade of SIL. The patients with high grade intraepithelial lesions (HSIL) usually have persistent high risk HPV (16 and 18) and uncommonly regress. They usually progress to carcinoma if left untreated.[5]

Structure of HPV

Human papilloma viruses are the member of Papillomavirus family of DNA viruses. These are circular DNA viruses with 55 nm in diameter, and consist of an outer protein capsid and inner circular genome. The circular genome contains 8,000 nucleotides and encodes 8 viral proteins. HPV genome contains three regions (**Fig. 8.1**):

1. *An upstream regulatory region* (URR) (also known as non-coding region): It contains the viral promoter that control viral transcription and replication.
2. *An early (E) region*: E region contains open reading frames (ORF) and encodes nonstructural proteins, such as E1, E2, E4, E5, E6 and E7. These proteins are involved in viral replication, transcription and transformation. They also help in viral adaptation in changing environment. E1 is the viral replication protein and E2 protein controls viral transcription and replication. E2 also interacts with viral oncogenes E6 and E7 and helps in carcinogenesis.
3. *A late (L) region*: L region encodes viral capsid proteins L1 and L2 that are expressed late in viral life. The capsid proteins form the structure of the virus and facilitate in DNA maturation and packaging.

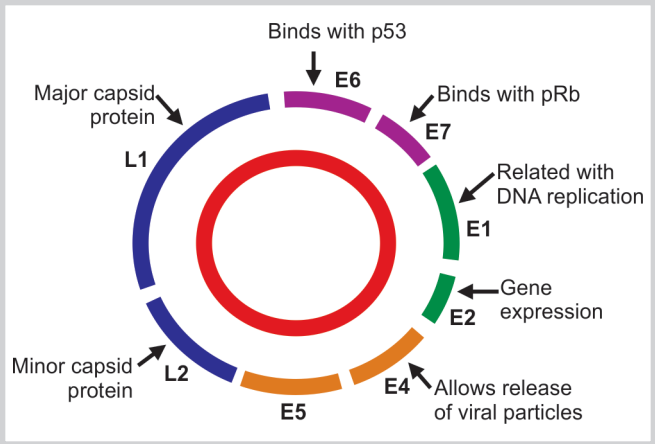


Fig. 8.1: Schematic diagram of human papilloma virus (HPV) structure shows an outer protein capsid and inner circular genome. The circular genome contains 8,000 nucleotides and encodes 8 viral proteins. Early region (E) encodes nonstructural proteins such as E1, E2, E4, E5, E6 and E7. Late region (L) encodes viral capsid proteins L1 and L2 that are expressed late in viral life

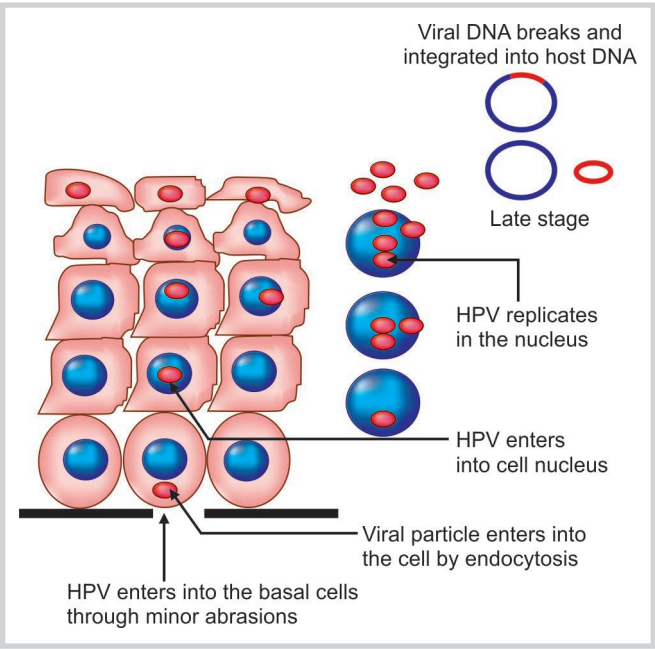


Fig. 8.2: Schematic diagram shows the life cycle of human papilloma virus (HPV) infection. In the early stage of viral infection, HPV virus remains within the nucleus as episome and proliferates within the nucleus. In late stage, the viral DNA integrates within the host DNA. Viral E2 genomic portion is lost and that promotes over expression of oncoproteins E6 and E7

Viral Life Cycle

During sexual intercourse, HPV enters into the female genital tract by small superficial abrasions. The virus particles are attached within the basement membrane. Within a few hours, the virus particles enter within the basal layer of the epithelial cell by endocytosis (**Fig. 8.2**). In the epithelial cells, the envelope protein of the virus is decomposed and viral DNA is routed towards

the host DNA nucleus. In the nucleus of the epithelial cells, the viral particle remains as episome and the virus is not integrated within the host DNA. No cytological abnormality is noted in this stage.[6] The viral episomes are maintained in the basal cells. One daughter basal cell retained the basal characteristics and other daughter cell differentiates with the viral episomes. The viral assembly takes place in the mature squamous epithelium and amplified viruses are released. This takes usually three weeks that corresponds to the maturation time of basal cells to squamous cells. Uncommonly, HPV infection persists and viral DNA integrates within the host DNA. The circular E2 becomes linear and is incorporated within host DNA. Loss of the function of E2 permits over expression of oncoproteins E6 and E7 (**Fig. 8.3**).

Viral Protein Interaction and Oncogenic Transformation

Human papilloma virus DNA is usually extra chromosomal or episomal in benign proliferative lesions of cervix. During persistent infection, HPV DNA integrates within the host DNA. There is breakage in E1/ E2 region and loss of E1 and E2. Loss of E2 leads to uncontrolled and uninhibited expression of E6 and E7 oncoproteins.

E6 Protein

E6 protein interacts with several host cell proteins (**Box 8.1**). E6 particularly binds with tumor suppressor protein p53 and inhibits the function of p53. E6 also binds with E6-associated protein, BAK, Paxilin, p16 (INK4A), etc. The overall effects of E6 are immortalization of cell, telomerase activation, chromosomal destabilization and anti-apoptosis.[7,8]

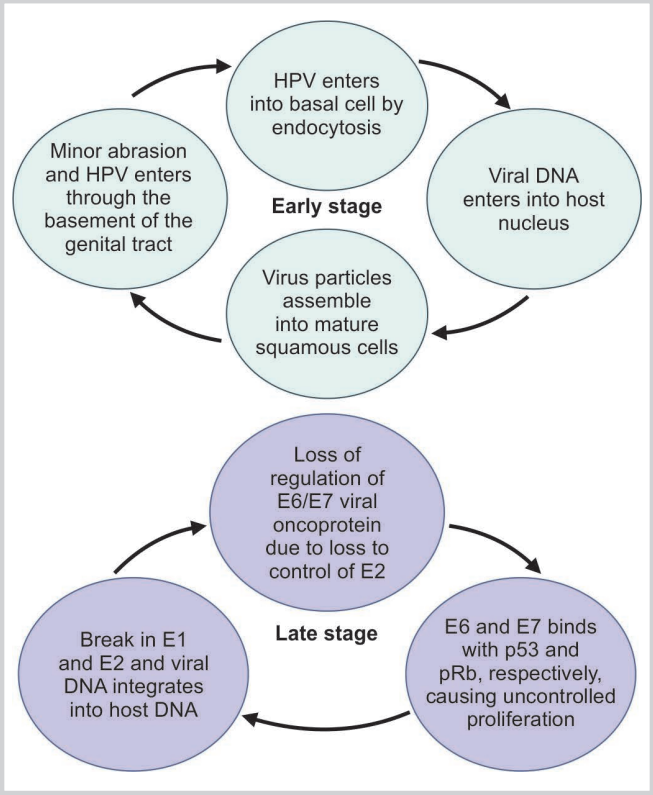


Fig. 8.3: This diagram highlights the major events in the human papilloma virus (HPV) infection

E7 Protein

E7 protein also binds with several host proteins particularly retinoblastoma protein (pRb) and its related pocket proteins. Binding of E7 with pRb releases transcription factor E2F. Released E2F facilitates cell cycle progression.[7-9] E7 protein also binds with E2F cyclin A complex, histone H1 kinase, cyclin E and TATA box binding protein.

E4 Protein

E4 gene is a part of early region. This E4 protein is necessary for viral maturation. E4 protein interacts with the cytoskeletal structure of the host epithelial cells and permits HPV to leave the cell. E4 and cytoskeleton interaction is responsible for koilocytotic changes. E4 protein interacts with the mitotic spindle formation and cytokinesis and therefore causes multinucleation.[7-9]

E5 Protein

E5 protein facilitates the interaction of growth factor receptors and ligands of the cell and therefore enhances cellular growth.

CERVICAL PRENEOPLASTIC LESIONS

Dysplasia

Reagan et al., in the year 1953, introduced the term dysplasia to indicate disordered maturation of the squamous epithelium.[10] In 1975, World Health Organization proposed unified terminology to describe precursor lesions of cervical carcinoma. Dysplasia was graded as mild, moderate and severe form.[11] In this classification, carcinoma in situ was considered as distinct entity from severe dysplasia rather than a spectrum of a single disease process with variable severity.

BOX 8.1 **Viral early protein functions**

E2: E2 protein controls viral transcription and replication
E1: Helps in the viral replication protein
E4:
- Interacts with the cytoskeletal structure of host
- Interacts with the mitotic spindle formation and cytokinesis

E6: Binds with tumor suppressor protein p53 and inhibits the function of p53
E7: Binds with several host proteins particularly retinoblastoma protein and releases transcription factor E2F

Cervical Intraepithelial Neoplasia

Richart in 1981 introduced the term cervical intraepithelial neoplasia (CIN) to avoid confusion between severe dysplasia and carcinoma in situ.[12] The CIN terminology graded the preneoplastic lesions into three categories, CIN 1, CIN 2, and CIN 3, with carcinoma in situ being incorporated into the CIN 3 category. Therefore, this new terminology of CIN replaced arbitrary differentiation between severe dysplasia and carcinoma in situ. CIN is graded depending on the amount of undifferentiated cells from basement membrane to the superficial squamous epithelium. Grade 1 is labeled when up to one-third of basal layer is involved. When more than one-third to two-thirds is involved the grade is labeled as 2 and when more than two-thirds is involved, the grade is labeled as 3 (**Figs 8.4** to **8.7**).

Bethesda System

Currently, Bethesda system of reporting is the most popular and widely used. Bethesda system is a two-tiered system and classified the squamous intraepithelial lesions as LSIL and HSIL.

1. Low grade squamous intraepithelial lesions: LSIL encompasses both HPV-induced changes and cervical intraepithelial lesions grade 1.

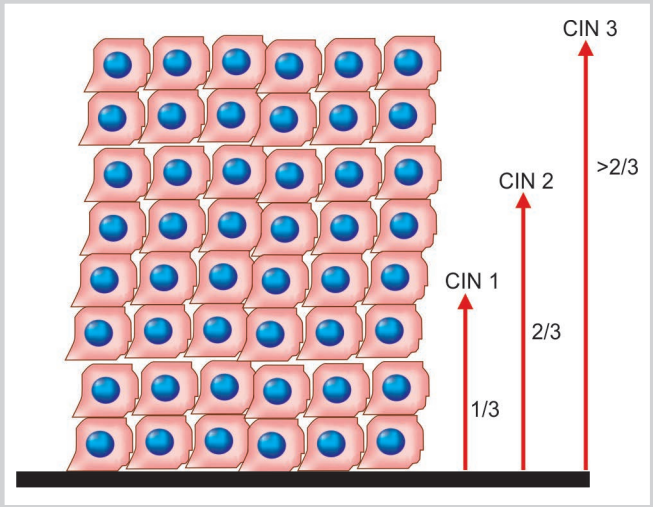


Fig. 8.4: Schematic diagram shows cervical intraepithelial neoplasia (CIN) 1, 2 and 3. CIN 1 involves up to one-third of the basal layer, CIN 2 involves one-third to two-third of the epithelium and CIN 3 involves more than two-thirds of the epithelium

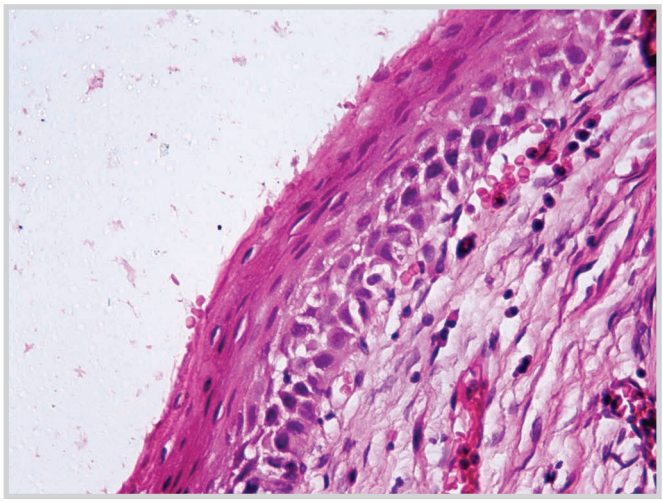


Fig. 8.5: Histology section of cervical intraepithelial neoplasia 1(CIN 1) involves less than one-third of the basal epithelium (H & E X MP)

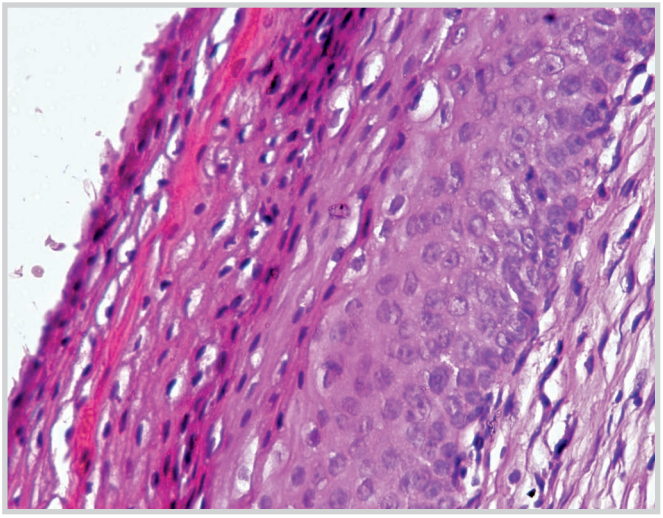


Fig. 8.6: Histology section of cervical intraepithelial neoplasia (CIN) 2 involves less than two-thirds of the epithelium (H & E X MP)

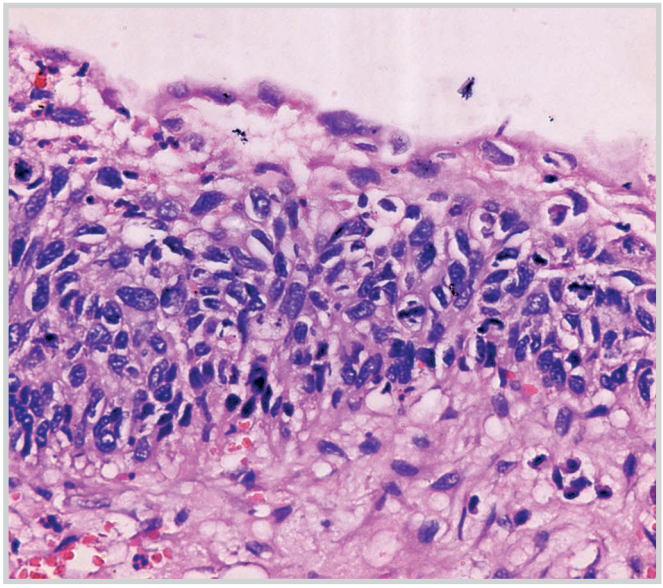


Fig. 8.7: Histology section of cervical intraepithelial neoplasia 3 (CIN 3) involves full thickness of the epithelium (H & E X MP)

TABLE 8.2: Nomenclature of cervical pre neoplastic lesions

Classification	*Grades*				
WHO	Mild dysplasia	Moderate dysplasia	Severe dysplasia	Carcinoma in situ	Carcinoma
CIN	CIN 1	CIN 2	CIN 3	CIN 3	Carcinoma
Bethesda	LSIL	HSIL	HSIL	HSIL	Carcinoma

Abbreviations: CIN—cervical intraepithelial neoplasia; HSIL—high grade squamous intraepithelial lesion; LSIL—low grade squamous intraepithelial lesion; WHO—World Health Organization

2. High grade intraepithelial lesions: HSIL includes cervical intraepithelial lesions grade 2, grade 3 and also carcinoma in situ.

Bethesda system closely reflects the biology of cervical pre neoplastic lesions and unifies the cervical cytology and histology reporting. **Table 8.2** shows the comparison of different classification of cervical preneoplastic lesions.

Low Grade Squamous Intraepithelial Lesion

Low grade squamous intraepithelial lesion (**Box 8.2**) is caused by both low and high risk HPV. In fact, many LSIL regresses if not treated and approximately 21% LSIL progresses to HSIL. Only 1% of LSIL cases may progress to invasive cancer, if left untreated.

Cytology

Smears show abnormality of superficial and intermediate squamous cells. The cells are usually present singly or in small sheets. There is mild nuclear enlargement and the nucleus occupies one-third of the cytoplasmic area (**Fig. 8.8**). The nucleocytoplasmic ratio is usually low (**Fig. 8.9**). There is mild nuclear enlargement and hyperchromasia with irregular nuclear contour (**Fig. 8.10**). Chromatin is finely granular. Nucleoli are either inconspicuous or absent. Koilcyotsis are seen in LSIL cases (**Fig. 8.11**). The cells show large perinuclear halo with clear distinct thick cytoplasmic margin (**Fig. 8.12**). Nuclei are always enlarged in koilocytotic cells (**Fig. 8.13**).

Differential Diagnosis of LSIL

1. *Reactive inflammatory changes in squamous epithelial cells:* Nuclear enlargement is often seen in squamous cells due to the effect of inflammation. However, other nuclear abnormalities are not noted in these cells.
2. *Air drying artifact:* Air drying may cause nuclear enlargement and may be mistaken as LSIL.
3. *Nonspecific perinuclear halos:* Nonspecific perinuclear halos may be seen in inflammation or *Trichomonas* infection. No nuclear atypia is seen in these conditions.
4. *Reactive changes in endocervical cells*: Reactive endocervical cells may have enlarged nucleus and polygonal shape. These cells may mimic LSIL. However, accompanying more recognizable endocervical cells may indicate their character.
5. *Atypical Squamous Cells of Undetermined Significance (ASCUS)*: It is often difficult to distinguish ASCUS from LSIL cases.

BOX 8.2 **Low-grade squamous intraepithelial lesion**

- Single or sheets of superficial and intermediate cells.
- Abundant cytoplasm
- Nucleus
  - Enlarged nucleus: Nucleus occupy more than one-third of the cytoplasmic area (3 to 5 times of a normal intermediate cell nucleus)
  - Mild hyperchromasia
  - Irregular margin
  - Evenly distributed fine granular chromatin
  - Nucleoli absent
- Low nucleocytoplasmic (N/C) ratio
- *Koilocyosis:* Distinct perinuclear cytoplasmic vacuoles with sharp condensed thick cytoplasmic margin. Nuclei are enlarged.

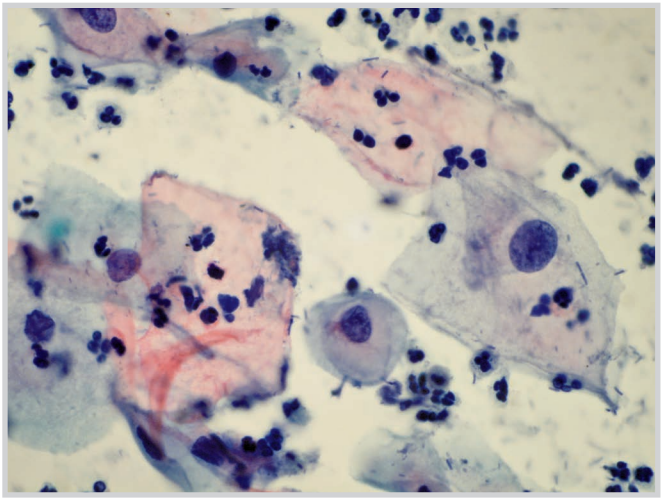


Fig. 8.8: Cervical smear of low grade squamous intraepithelial lesion (LSIL) showing enlarged nucleus occupying more than one-third of the cytoplasmic area (Papanicolaou's stain X HP)

Management of LSIL cases: Follwing are the point of management for LSIL cases

- Colposcopy is recommended in LSIL cases
- If the patient is pregnant, then colposcopy could be deferred up to 6 weeks of pregnancy
- If there is no lesion in colposcopy, unsatisfactory colposcopy, or the patient is not pregnant then endocervical sampling is preferred

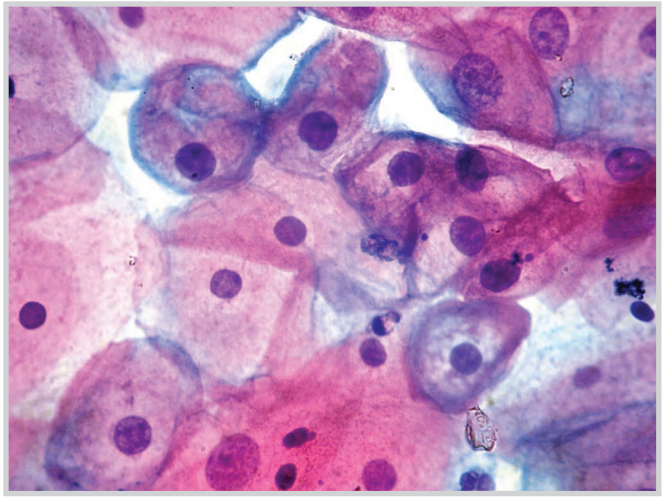


Fig. 8.9: Cervical smear of low grade squamous intraepithelial lesion (LSIL) showing mild enlargement of the nucleus and altered nucleocytoplasmic ratio (Papanicolaou's stain X HP)

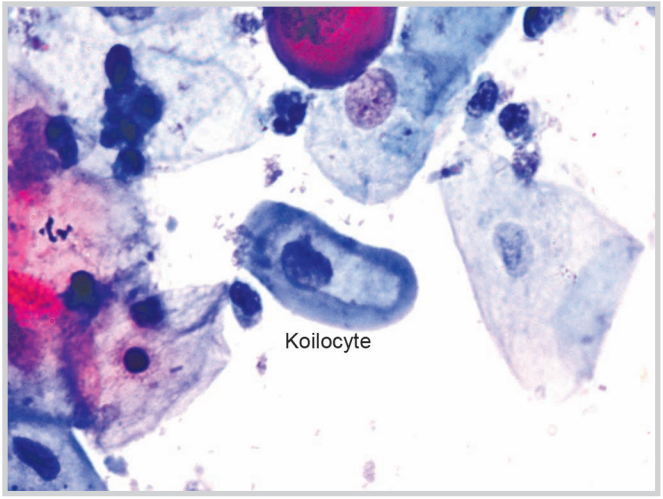


Fig. 8.12: Koilocytes in a case of low grade squamous intraepithelial lesion (LSIL) showing large perinuclear halo with clear distinct thick cytoplasmic margin (Papanicolaou's stain X HP)

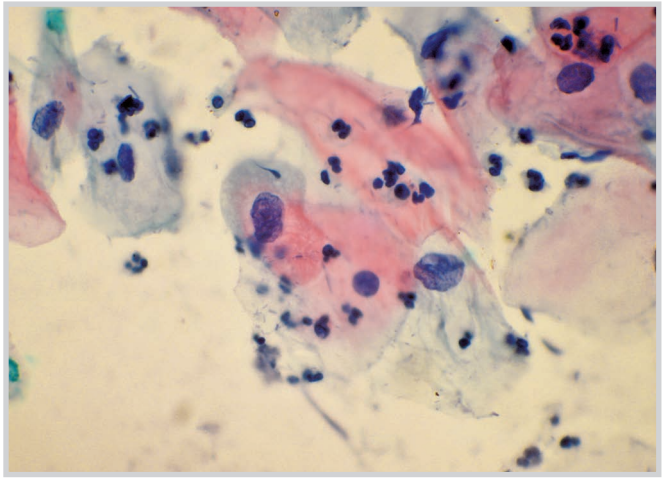


Fig. 8.10: Cervical smear of low grade squamous intraepithelial lesion (LSIL) showing irregular nuclear margin (Papanicolaou's stain X HP)

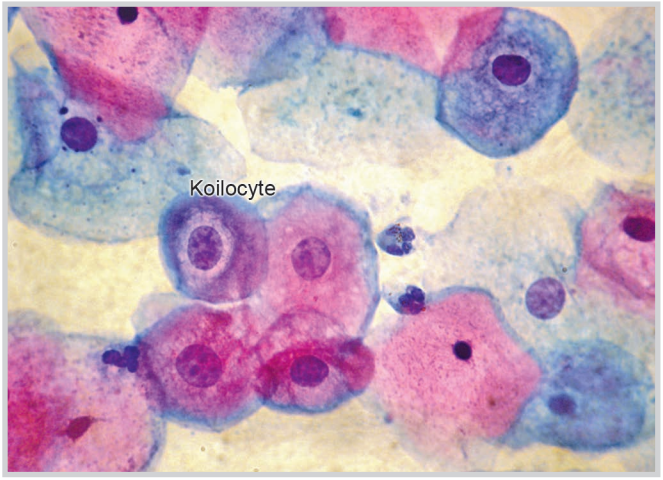


Fig. 8.13: Koilocytes in a case of low grade squamous intraepithelial lesion (LSIL) with enlargement of the nucleus (Papanicolaou's stain X HP)

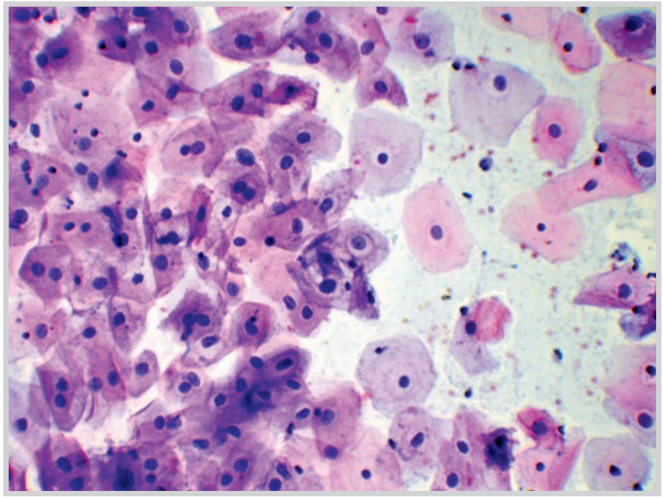


Fig. 8.11: Cervical smear of low grade squamous intraepithelial lesion (LSIL) showing many koilocytes (Papanicolaou's stain X MP)

- If colposcopy does not show CIN 2 or 3 then HPV DNA testing or repeat cervical cytology should be done
- If the patient shows high-risk HPV DNA or any of the cytology shows atypical squamous cell or greater, then a repeat colposcopy is advised
- If colposcopic-guided biopsy shows CIN 2 or 3, then surgical ablation could be done
- In postmenopausal or adolescent patients, colposcopy could be deferred and repeat cytology testing at 6 monthly intervals can be done.

High Grade Squamous Intraepithelial Lesions

High grade intraepithelial lesion in Bethesda system includes CIN 2 and CIN 3 (moderate dysplasia, severe dysplasia and carcinoma in situ). Only 0.5% of all cervical smears show HSIL. High-risk HPV is found in 97% of HSIL cases.[13] HSIL usually develops in the endocervical epithelium either in the transformation zone or in the endocervical canal.

Cytology

Cells in HSIL (**Box 8.3**) are usually parabasal, basal or metaplastic type. They are arranged in small sheets, three-dimensional crowded groups or singly (**Figs 8.14** and **8.15**). Individual cells are round to oval with scanty cytoplasm and high N/C ratio (**Fig. 8.16**). Cytoplasm is dense or lacy in character. In case of keratinizing variant, there may be orangeophilia in the cytoplasm (**Fig. 8.17**). Nuclei of the cells are enlarged, moderately pleomorphic, hyperchromatic with coarse chromatin. Nuclear contour is markedly irregular (**Fig. 8.18**). Nucleoli are prominent. In some cases, HSIL may not show squamoid differentiation and the cells are round to oval with vacuolated cytoplasm mimicking glandular in origin (**Fig. 8.19**). In liquid-based cytology (LBC) preparation, the cells in HSIL cases are easily identifiable (**Figs 8.20** to **8.22**).

Table 8.3 compares the cytological differentiating points between HSIL and LSIL.

Differential Diagnosis

Following are the differential diagnosis for HSIL

- *Atrophic smear*: Small parabasal cells in atrophic smear may simulate HSIL at times. However, careful observation of nuclear character may be helpful in diagnosis.
- *Squamous metaplastic cells*: Squamous metaplastic cells with relatively large nucleus and dense chromatin may pose diagnostic difficulties with HSIL.
- *Histiocytes*: Mononuclear histiocytes with enlarged nucleus may also simulate HSIL. However, nuclei of histiocytes have fine chromatin texture.
- *Atypical squamous cells cannot exclude high-grade squamous intra-epithelial lesion (ASC-H):* The presence of atypical squamous cells with nuclear enlargement and pleomorphism is extremely difficult to distinguish from HSIL.
- *Intrauterine device effect*: Small cells in IUD may pose diagnostic difficulty with HSIL.
- *Endometrial cells*: At times, clusters of endometrial cells may be difficult to distinguish from HSIL. However, HSIL clusters are less well circumscribed and individual cells are larger than endometrial cells.

Management of HSIL Cases[14]

- Colposcopy with endocervical assessment
- If colposcopy confirms CIN 2/3, then surgical excision is done

BOX 8.3 **High grade intraepithelial lesion**

- Single or sheets of cells
- Parabasal or basal type of cell
- Cytoplasm is dense or lacy and orangeophilic in keratinized variant
- Nucleus
  - Three times size of intermediate cell nuclei
  - Moderately pleomorphic
  - Hyperchromatic
  - Marked irregular nuclear membrane
  - Coarse chromatin
  - Prominent nucleoli
- Nucleo-cytoplasmic ratio is high

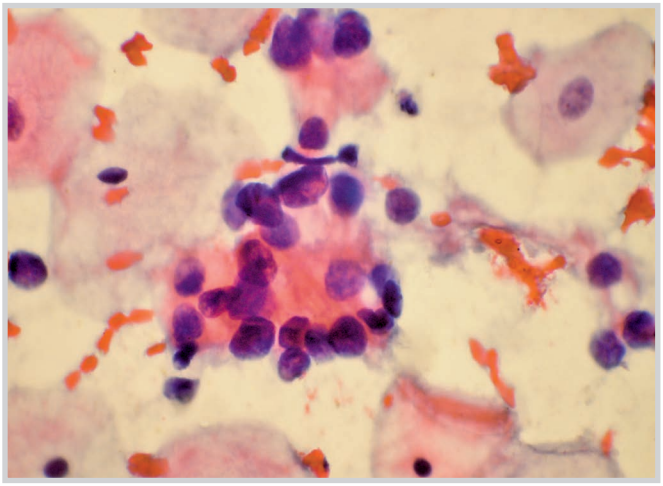


Fig. 8.14: Cervical smear in a high grade squamous intraepithelial lesion (HSIL) case showing small cluster of cells with overlapping nuclei (Papanicolaou's stain X HP)

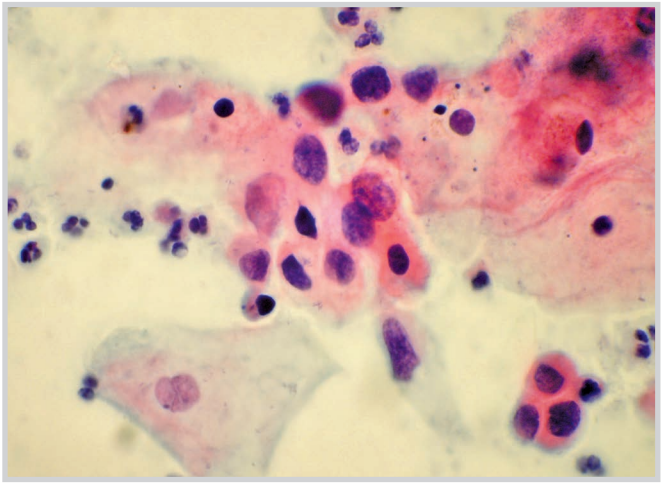


Fig. 8.15: Discrete dysplastic cells in high grade squamous intraepithelial lesion (HSIL) case (Papanicolaou's stain X HP)

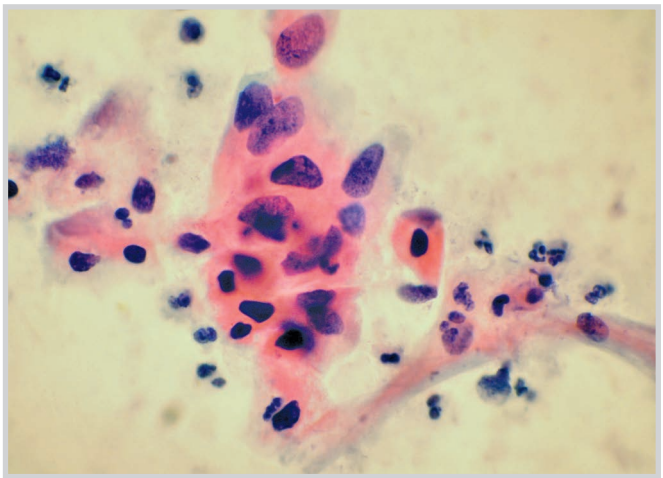


Fig. 8.16: Cervical smear of a high grade squamous intraepithelial lesion (HSIL) case shows enlarged nuclei occupying more than two-thirds of the cytoplasmic area (Papanicolaou's stain X HP)

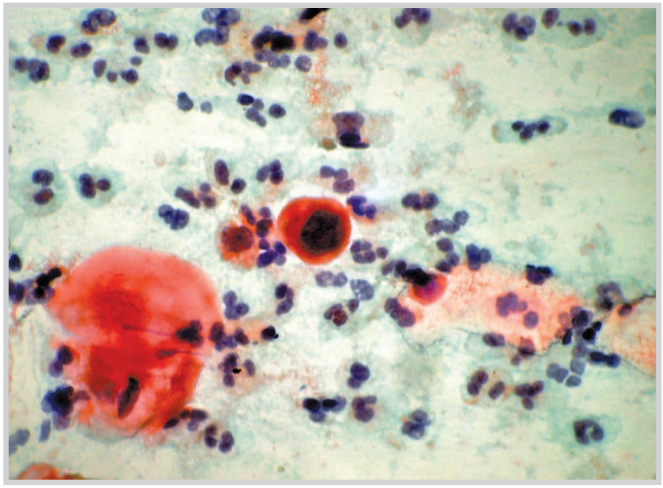


Fig. 8.17: Cervical smear in a high grade squamous intraepithelial lesion (HSIL) case showing orangeophilic cytoplasm (Papanicolaou's stain X HP)

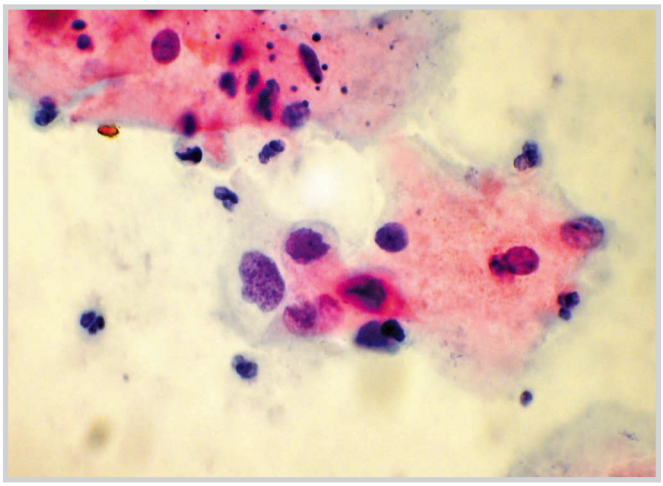


Fig. 8.18: Cervical smear in a high grade squamous intraepithelial lesion (HSIL) case showing nuclear margin irregularity (Papanicolaou's stain X HP)

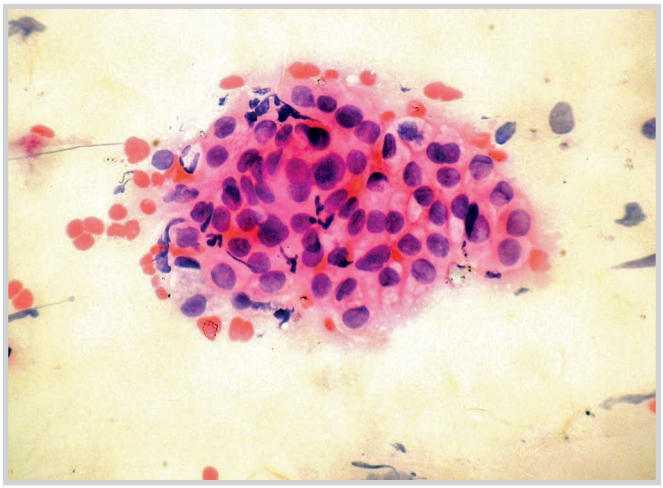


Fig. 8.19: Round to oval cells with vacuolated cytoplasm mimicking glandular in origin in a high grade squamous intraepithelial lesion (HSIL) case (Papanicolaou's stain X HP)

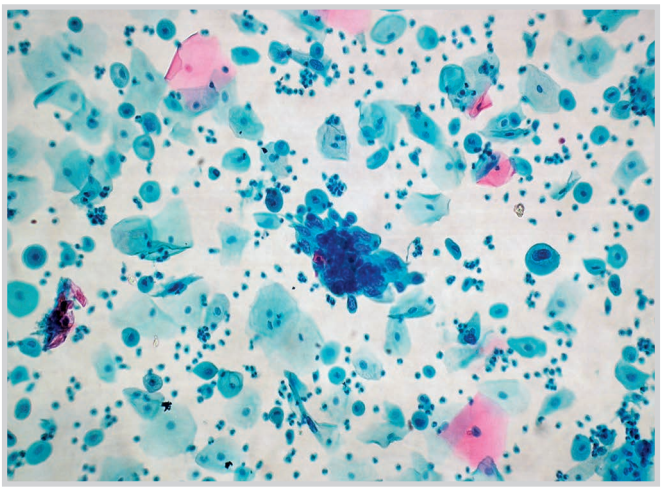


Fig. 8.20: High grade squamous intraepithelial lesion in liquid-based cytology (SurePath). Small cluster and discrete dysplastic cells (Papanicolaou's stain X MP)

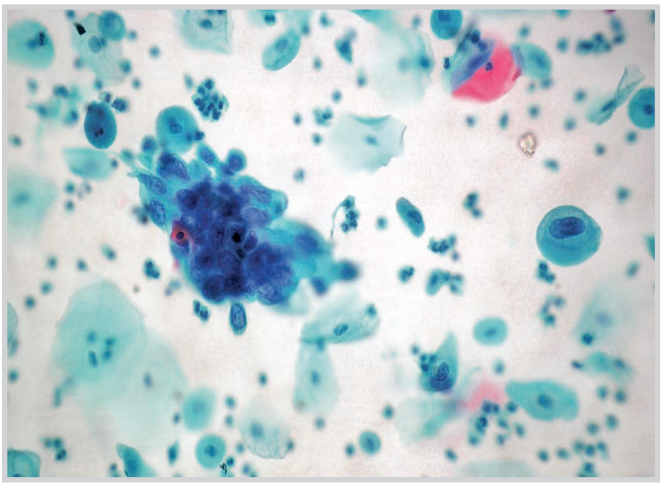


Fig. 8.21: High-powered view of the same in the high grade squamous intraepithelial lesion (HSIL) case (Papanicolaou's stain X HP)

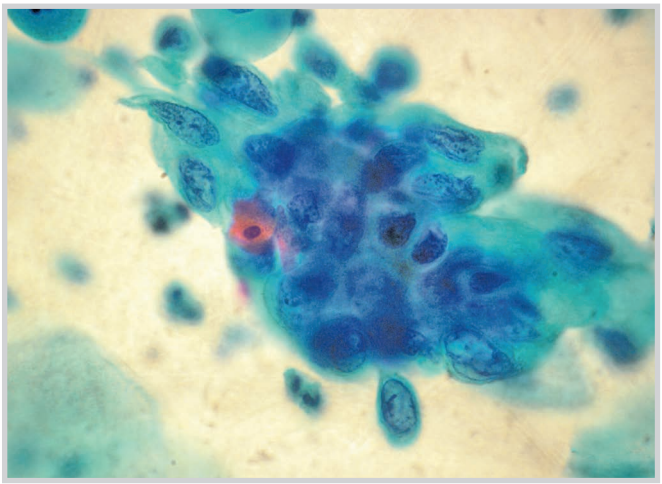


Fig. 8.22: Magnified view of the dysplastic cells showing marked nuclear enlargement and nuclear margin irregularity in high grade squamous intraepithelial lesion (HSIL) case (Papanicolaou's stain X OI)

TABLE 8.3: Comparison of LSIL and HSIL

Cytological features	*LSIL*	*HSIL*
Cell arrangement	Singly and in clusters	Singly and in clusters
Cell type	Superficial and intermediate	Parabasal, basal and metaplastic
Koilocytes	Present	Usually absent
Cell cytoplasm	More	Less
N/C ratio	Low	High
Nucleus		
Size	Mild enlargement	Moderate enlargement
Pleomorphism	Mild	Moderate
Contour	Mildly irregular	Moderately irregular
Hyperchromasia	Mild	Moderate
Chromatin	Fine	Coarse
Nucleoli	Absent	Present
Mitosis	Rare	Frequent

Abbreviations: HSIL—high grade squamous intraepithelial lesion; LSIL—low grade squamous intraepithelial lesion;

- If colposcopy is negative then either a diagnostic excisional biopsy or repeat PAP smear should be done.

ATYPICAL SQUAMOUS CELLS

The term ASCUS is used when cytology smear is suggestive of squamous intra-epithelial lesion (SIL) but there are not enough evidences to have a definitive interpretation of SIL. ASCUS terminology has evoked lot of confusion because of low reproducibility, heterogeneous mixture of diagnoses and variable outcomes.

Bethesda system divides atypical squamous cells as:
- ASCUS and
- ASC-H.

In TBS 2001, the term "ASCUS favors reactive" is eliminated from the reporting format.

ASCUS represents an equivocal case and a diagnosis of exclusion. The interpretation of ASCUS is subjective and depends on pathologist's diagnostic threshold. ASCUS should be reported as minimum as possible. There is no correct rate of ASCUS and the prevalence of ASCUS is inversely proportional with the reporting of LSIL. It is suggested that the rate of ASCUS diagnosis should be less than 5% of total cervical smear. The reporting rate of ASCUS in cervical smear should not exceed two to three times of SIL.

Cytology

Atypical squamous cell with mature intermediate type cytoplasm: This is the most commonly seen abnormality of ASCUS (**Box 8.4**). Here, ASCUS involves nuclear atypia in squamous cells with superficial and intermediate type of cytoplasm. The cells are polyhedral with rounded cytoplasmic border and thickened margin. Nuclear enlargement is 2 to 3 times that of a normal intermediate squamous cell (**Fig. 8.23**). Nuclei show regular margin with fine granular chromatin.

BOX 8.4 **Atypical squamous cells of undetermined significance (ASCUS)**

Cytology
- Superficial and intermediate type of cell show changes
- Cytoplasm may show vague perinuclear halos
- Mildly increased N/C ratio
- Nucleus mildly increased in size
- Mildly hyperchromatic nucleus

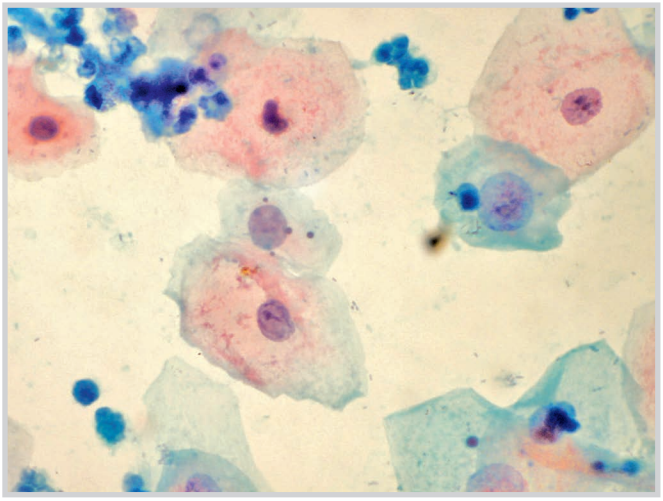


Fig. 8.23: Cervical smear of atypical squamous cell with mature intermediate type cytoplasm (Papanicolaou's stain X HP)

Atypical squamous cell in the setting of atrophy: There may be reactive and atypical changes in the background of atrophy (**Fig. 8.24**). The individual cells show nuclear enlargement, pleomorphism, hyperchromasia, irregular nuclear contour. The cytoplasm may be orangeophilic.[15] In doubtful cases, a short course of local estrogen therapy followed by repeat cervical smear may be helpful. Benign reactive changes associated with atrophy will resolve after estrogen therapy.

Atypical parakeratotic squamous cells: The individual cells are present singly or in small three-dimensional clusters. The cells are small polygonal with orangeophilic cytoplasm having dark nuclei (**Fig. 8.25**). Nuclei show irregular and angulated contour with irregularly distributed chromatin.[16]

ASCUS due to compromised specimen: This is the "*wastebasket*" category diagnosis and includes cases of poor fixation, preservation, obscuring material that compromise visualization and interpretation difficulties.

Reproducibility of ASCUS

Reproducibility of ASCUS is poor due to its high interobserver and intraobserver variability. Even after adequate experience or review of criteria by TBS atlas, the reproducibility of ASCUS is

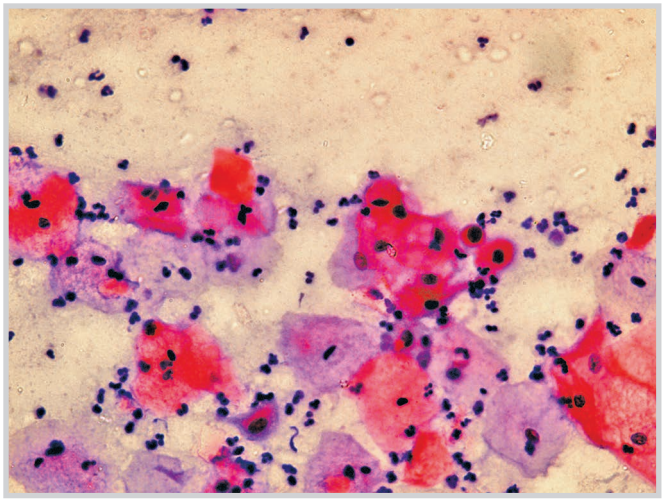


Fig. 8.24: Atypical changes in the background of atrophy in ASCUS case (Papanicolou's stain X HP)

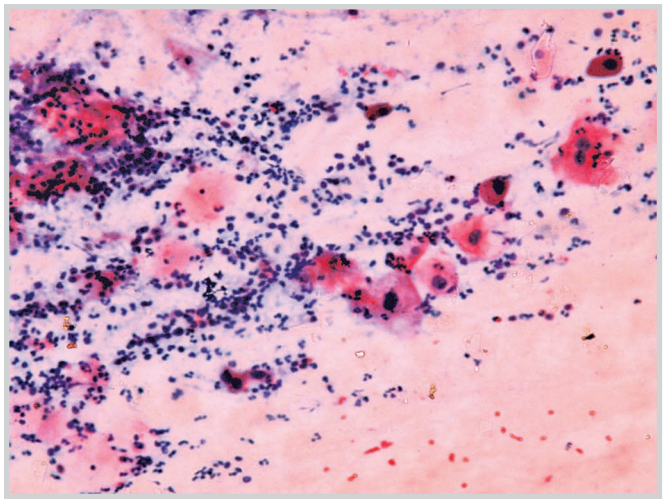


Fig. 8.25: Atypical parakeratotic squamous cells in an ASCUS case (Papanicolau's stain X HP)

poor. Complete consensus of ASCUS cases can be reached only in 30 % cases.[17,18]

Atypical squamous cell-high grade SIL cannot be excluded (*ASC-H*): ASC-H cases are suggestive of HSIL but fall short of cytological criteria for a definitive interpretation. ASC-H cases are less common and represent only 5–10 % of all ASC cases.[19] Subsequent HSIL is seen in 30–40% of cases of ASC-H.

Cytology (**Box 8.5**): Smears show usually small immature looking squamous cells (**Fig. 8.26**). Nucleus is enlarged, hyperchromatic with high N/C ratio and irregular contour (**Figs 8.27** and **8.28**). Small nucleoli may be seen.

Management of ASCUS Cases

Repeat cervical cytology, high-risk HPV DNA testing and colposcopy are recommended in case of ASC-US cases:

BOX 8.5 **ASC-H**

- Single and loose cohesive group of cells
- Immature small squamous cells
- Nucleus: mild to moderate enlargement, hyperchromasia and pleomorphism
- Increased nucleocytoplasmic ratio

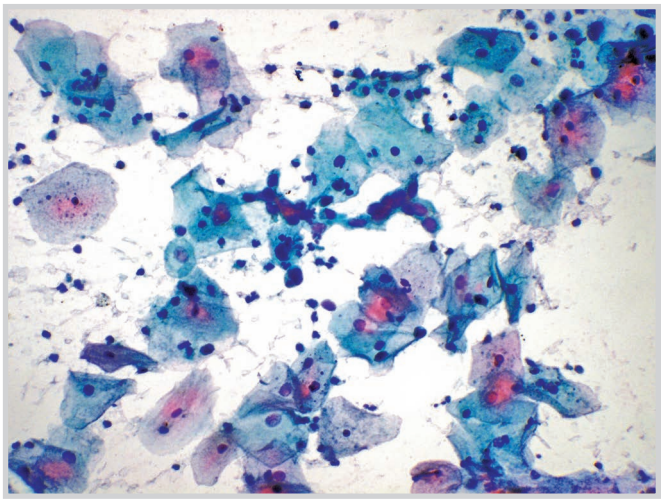


Fig. 8.26: Small immature-looking squamous cells in ASC-H case (Papanicolaou's stain X MP)

- If the patient is positive for high-risk HPV DNA then colposcopy is recommended
- If the woman is negative for high-risk HPV DNA then repeat cervical cytology at 6 monthly intervals should be done
- If two successive Pap test are negative then the patient can return to routine screening schedule
- If any of the two Pap test shows ASCUS or greater then colposcopy is recommended.

Management of ASC-H

- Colposcopy is always recommended in ASC-H patient
- If histology of the case does not show CIN 2 or 3 then repeat cervical smear or HPV DNA test for high risk viruses should be done
- If the patient shows ASC-H in repeat smear or high-risk HPV DNA positive then repeat colposcopy is advised.

SQUAMOUS CELL CARCINOMA

Squamous cell carcinoma (SQC) is the most common malignant tumor of the cervix. It occurs between 40 and 55 years of age. Early invasive carcinoma is asymptomatic, however, as the progression of carcinoma, the patient presents with vaginal bleeding and post coital or intermenstrual bleeding. In late invasive cases, the patient may show features of infiltration into

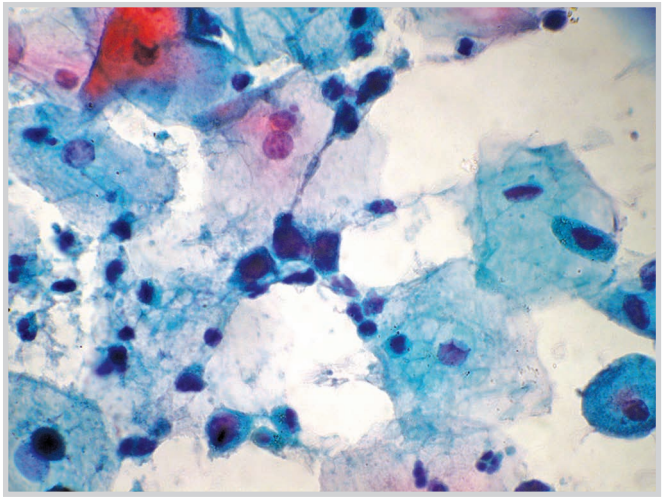


Fig. 8.27: Small cluster of cells with enlarged hyperchromatic nuclei in ASC-H case (Papanicolaou's stain X HP)

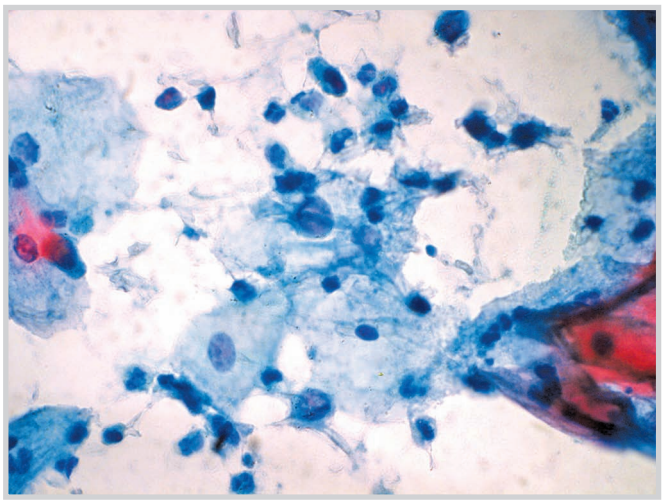


Fig. 8.28: Discrete dysplastic cells with enlarged nuclei in ASC-H case (Papanicolaou's stain X HP)

the surrounding tissue, such as back pain, hematuria, rectal bleeding and obstructive uropathy.

Squamous cell carcinoma of cervix is subtyped according to cell type and degree of differentiation as:

- Large cell non-keratinizing carcinoma: The tumor cells show individual cell kertinization. No squamous pearls are seen
- Keratinizing carcinoma: Keratin pearls are seen (**Fig. 8.29**)
- Small cell non-keratinizing carcinoma: Individual cells are small with scanty cytoplasm. No keratin pearls are seen.

WHO recommends two-tiered system of classification of squamous cell carcinoma of cervix:

- Keratinizing squamous cell carcinoma
- Non-keratinizing squamous cell carcinoma.

The other variants of squamous cell carcinoma of cervix are:

- Basaloid
- Verrucous
- Lymphoepithelioma like

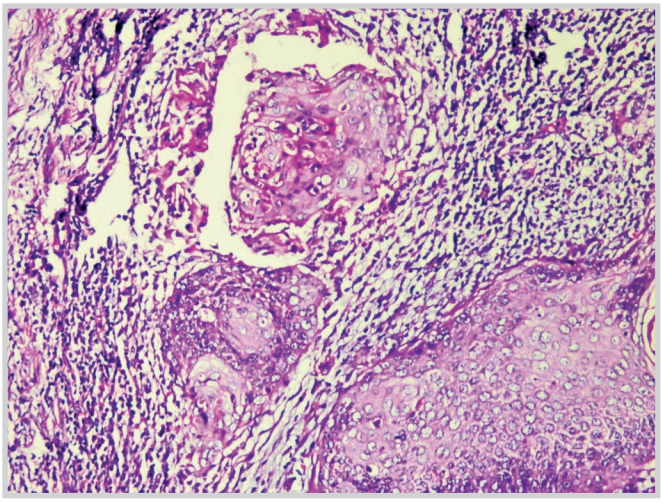


Fig. 8.29: Histology section of keratinizing squamous cell carcinoma of cervix showing Keratin pearls (H & E X MP)

- Papillary
- Condylomatous.

Cytology (Box 8.6)

Smear show discrete and small groups of malignant cells (**Fig. 8.30**). Individual cells show moderately pleomorphic, hyperchromatic nuclei with irregular clumped chromatin (**Fig. 8.31**). Cytoplasm of the cell is orangeophilic with well-defined border. The characteristic tadpole cells and fiber cells are noted. Tadpole cells are elongated in shape with large abnormal nuclei in one corner. Fiber cells are spindle-shaped with elongated hyperchromatic nucleus (**Fig. 8.32**).

Background tumor diathesis is an important feature of invasive carcinoma. Smear shows nuclear debris, RBCs and small necrotic tissue fragments (**Fig. 8.33**). Atrophic smear and heavy menstrual blood may simulate similar pattern. In LBC, the smear may show more clean background, however the typical malignant cells are easily recognized (**Figs 8.34A and B**). Careful observation may reveal tumor diathesis and other salient features of squamous cell carcinoma (**Figs 8.35A to D**).

Differential Diagnosis

- *HSIL:* It is the closest differential diagnosis of squamous cell carcinoma. Tumor diathesis is the most important differentiating feature between HSIL and invasive carcinoma. However, in the absence of tumor diathesis, it may be difficult to distinguish invasive carcinoma from HSIL.
- *Atypia of repair*: Marked reparative changes may mimic invasive carcinoma. Cells may show moderate nuclear enlargement and pleomorphism with large prominent nucleoli in reparative changes. However, nuclear chromatin is fine and granular in reparative cells in comparison to coarse irregular clumped chromatin in carcinoma.
- *Atrophic smear:* Dirty background along with cells having orangeophilic cytoplasm and moderately enlarged dark

BOX 8.6 Squamous cell carcinoma

Keratinizing
- Scattered and small clusters of tumor cells
- Moderately pleomorphic cell with high N/C ratio
- Nuclei
  - Pleomorphic
  - Hyperchromatic
  - Irregular coarse chromatin
  - Nucleoli present
- Cytoplasm: dense orangeophilic
- Tadpole cells: Elongated cell with large abnormal nuclei in one corner
- Fiber cell: Spindle-shaped hyperchromatic nuclei
- Tumor diathesis: Dirty background with necrotic tissue fragments and RBCs

Nonkeratinizing
- Cells are smaller
- Cytoplasm: Basophilic
- Prominent nucleoli

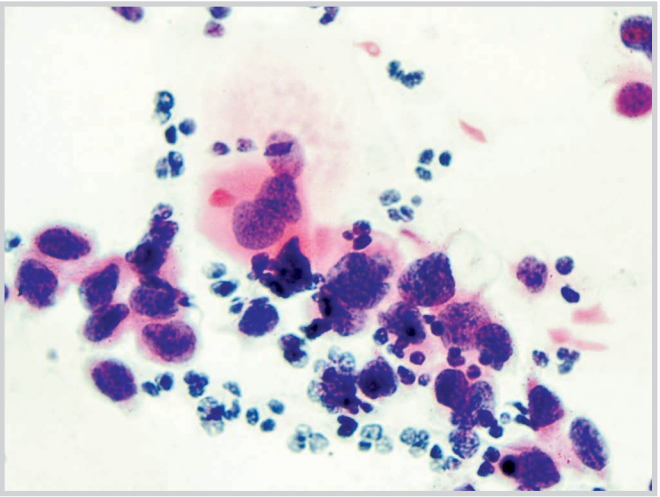


Fig. 8.31: Smears show moderately pleomorphic, hyperchromatic nuclei with irregular clumped chromatin in squamous cell carcinoma of cervix (Papanicolaou's stain X HP)

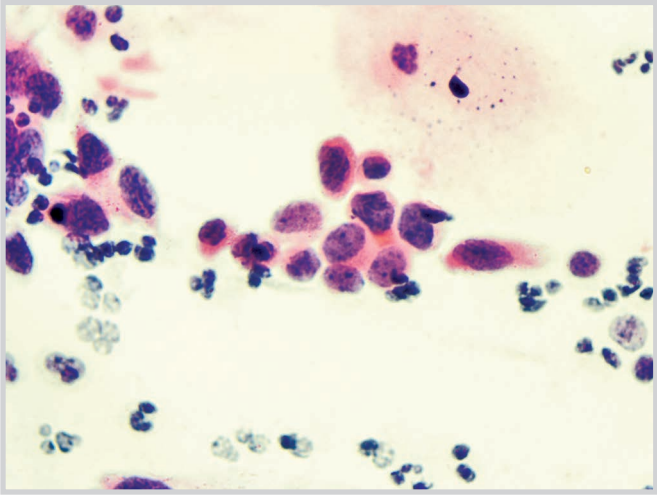


Fig. 8.30: Cervical smear showing discrete and small groups of malignant cells in squamous cell carcinoma of cervix (Papanicolaou's stain X HP)

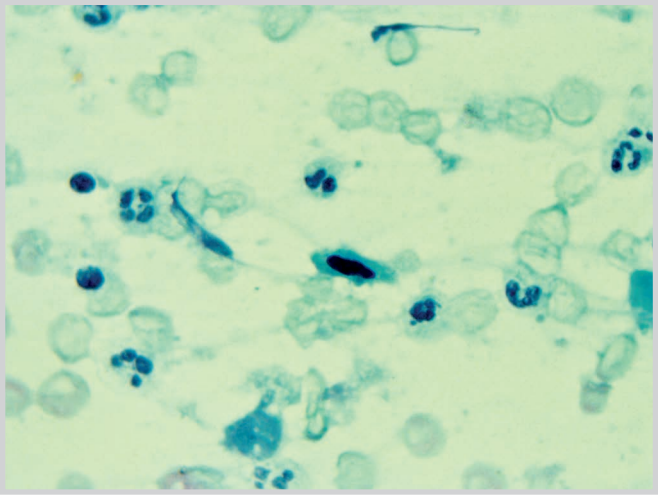


Fig. 8.32: Fiber cell with spindle-shaped elongated hyperchromatic nucleus in squamous cell carcinoma of cervix (Papanicolaou's stain X HP)

nuclei of atrophic smear may often mimic invasive cancer. However, chromatin pattern of these cells is usually smudgy and help in differentiating from carcinoma.
- *Endometrial cells*: Small spherical clusters of endometrial cells in a menstrual background may simulate small cell non-keratinizing squamous cell carcinoma. A careful history of growth in cervix may be helpful in this condition.

ADENOCARCINOMA

Endocervical adenocarcinoma comprises of 15 % of all cervical carcinomas.[20] The incidence of adenocarcinoma of cervix has increased about 5–20% during the last few decades. In contrast to SQCs which are associated with HPV 16, adenocarcinomas of cervix are more frequently related with HPV 18.[21] Mean age of the patient is 55 years. Majority of the patients are asymptomatic. However, some patients may present with vaginal bleeding, discharge and pain.

Cytology

Cytology (**Box 8.7**) smear of the endocervical adenocarcinoma usually shows abundant malignant cells. The cells are present discretely or in small loose clusters (**Fig. 8.36**). The margin of the cluster shows scalloped border. There is overcrowding of the nuclei in the cluster (**Fig. 8.37**). Individual cells show round to oval nucleus with moderate amount of vacuolated cytoplasm (**Fig. 8.38**). Nuclei show moderate pleomorphism with coarse chromatin and large prominent nucleoli (**Figs 8.39** and **8.40**). In addition, smears show tumor diathesis. However, in LBC preparation, the cells are usually discrete in a clear background (**Fig. 8.41**).

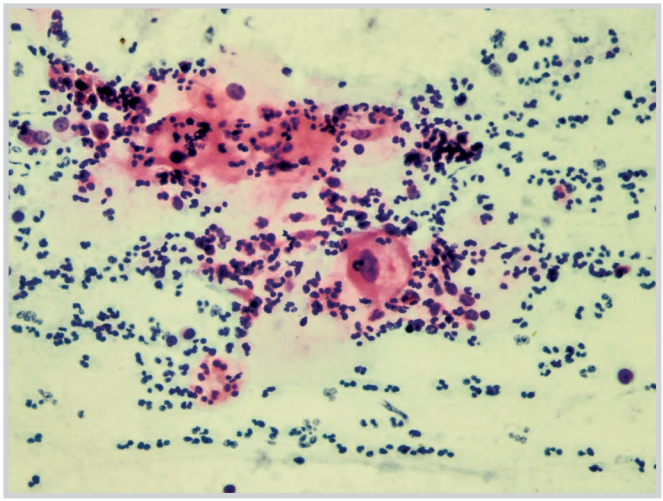
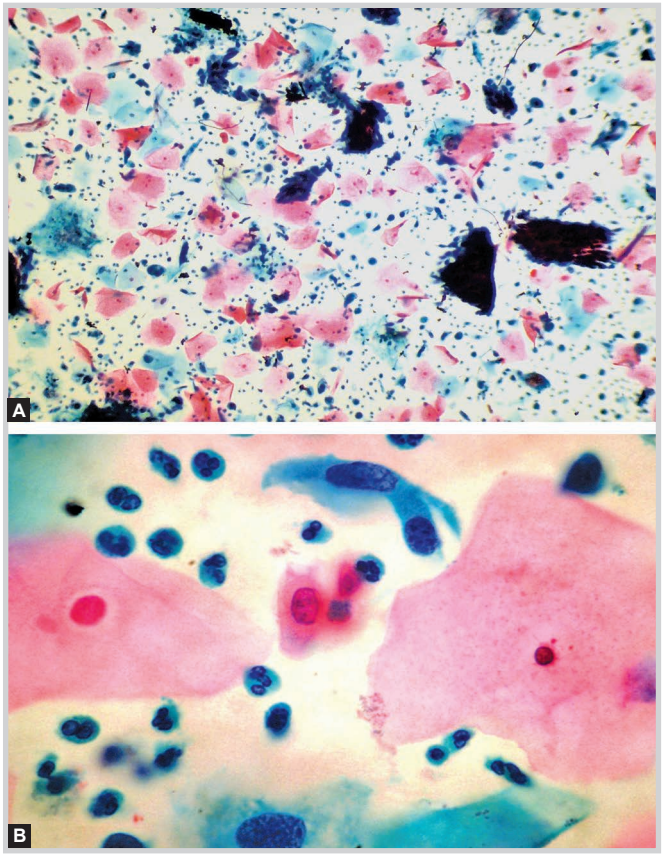


Fig. 8.33: Tumor diathesis with nuclear debris, red blood cells (RBCs) and small necrotic tissue fragments in invasive squamous cell carcinoma of cervix (Papanicolaou's stain X HP)



Figs 8.34A and B: (A) Liquid-based cytology (LBC) preparation showing clusters and discrete malignant cells with clean background in squamous cell carcinoma of cervix (Papanicolaou's stain X LP); (B) LBC preparation showing distinct malignant cells in squamous cell carcinoma of cervix (Papanicolaou's stain X HP).

Differential Diagnosis

- *Reactive endocervical cells*: Reactive endocervical cells with n♥uclear pleomorphism may simulate as endocervical adenocarcinoma. However, cells are in monolayer sheets and other nuclear features of adenocarcinoma are absent.
- *Microglandular hyperplasia*: Reactive endocervical cells in microglandular hyperplasia may often mimic as endocervical adenocarcinoma. Nuclear enlargement, pleomorphism and cytoplasmic vacuoles may mislead the cytologist. A careful history (such as history of pregnancy or the presence of endocervical polyp) may be needed in such case.
- *Endometrial adenocarcinoma*: If possible, the endometrial adenocarcinoma should be differentiated from endocervical adenocarcinoma. Endometrial adenocarcinoma is usually seen in post menopausal patient. The cells of endometrial carcinoma are often in tight spherical clusters and discrete along with many histiocytes in the background (**Table 8.4**)

ENDOMETRIAL ADENOCARCINOMA

Most of the endometrial adenocarcinoma patients are postmenopausal and majority of the patients present with postmenopausal bleeding. Cervical smear is not a screening test of endometrial adenocarcinoma**.**

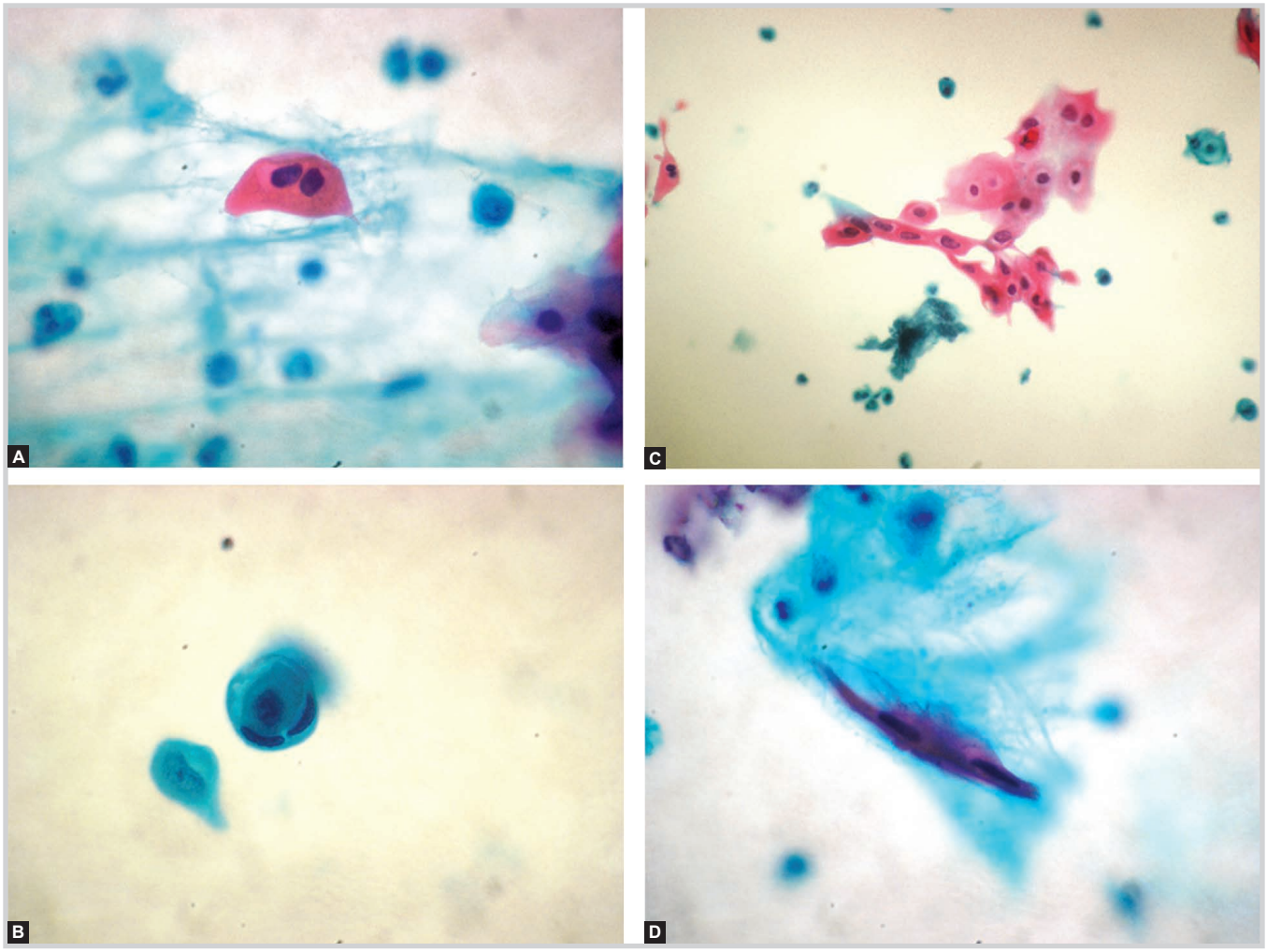
Cytology

Cytology (**Box 8.8**) smear of endometrial cells show tight clusters and discrete tumor cells (**Fig. 8.42**). The nuclei in the clusters show loss of polarity (**Fig. 8.43**). There may be numerous histiocytes in the smear and less number of malignant cells. Individual cells show nuclear enlargement, hyperchromasia and pleomorphism. Nucleoli are prominent. Cytoplasm of the cells often shows vacuoles. In addition, background of the smear shows finely granular tumor diathesis. In fact, the cytological features of endometrial carcinoma depend predominantly on the grade of endometrial adenocarcinoma. Relative abundance of cells increases from lower to higher grade of tumor. Similarly, nuclear enlargement and pleomorphism increase in severity depending on the grade of the endometrial carcinoma.

Atypical Glandular Cell

Atypical glandular cell (AGC) is defined as atypical cell with nuclear atypia more than reactive or reparative changes, however, fall short of unequivocal diagnosis of adenocarcinoma. AGC is less common than ASCUS and comprises of less than 1% of all abnormal cervical smear.[22] According to TBS 2001, AGC should always be specified as:

- AGC of endocervical in origin
- AGC of endometrial in origin
- AGC favor neoplasia (endocervical or endometrial).



Figs 8.35A to D: (A) Liquid-based cytology (LBC) preparation showing discrete malignant cells in squamous cell carcinoma of cervix (Papanicolaou's stain X HP); (B) Scanty tumor diathesis may be seen along with malignant cells (Papanicolaou's stain X HP); (C) Smear of LBC preparation shows characteristic squamous pearl (Papanicolaou's stain X OI); (D) Long slender fiber cell in LBC smear preparation (Papanicolaou's stain X OI)

BOX 8.7 **Adenocarcinoma**

- High cellularity
- Single and loose clusters of cells
- Well-defined scalloped borders of the cluster
- Columnar to cuboidal cells
- Nucleus
  - Large and round
  - Nuclear overlapping
  - Prominent nucleoli
  - Macronucleoli may be present
  - Coarse chromatin
- Cytoplasm: abundant and vacuolated
- Tumor diathesis present

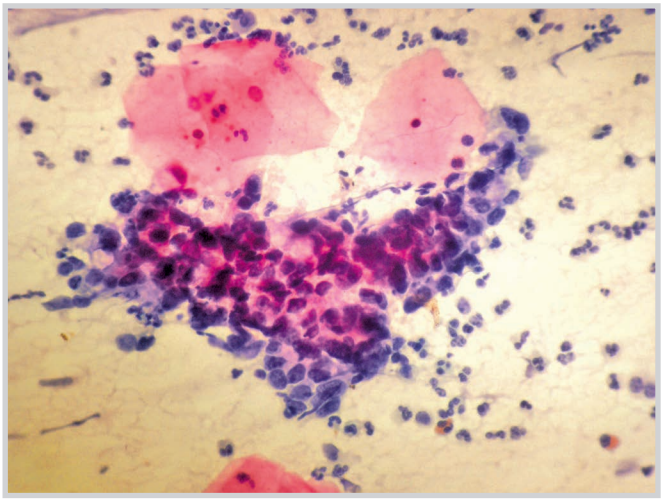


Fig. 8.36: Irregular small cluster of malignant cells in adenocarcinoma of cervix (Papanicolaou's stain X HP)

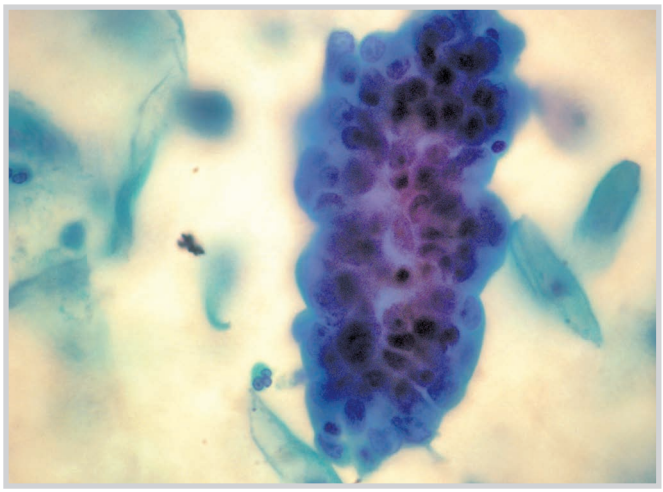


Fig. 8.37: Overcrowding of the nuclei in the cluster of malignant cells in adenocarcinoma of cervix (Papanicolaou's stain X HP)

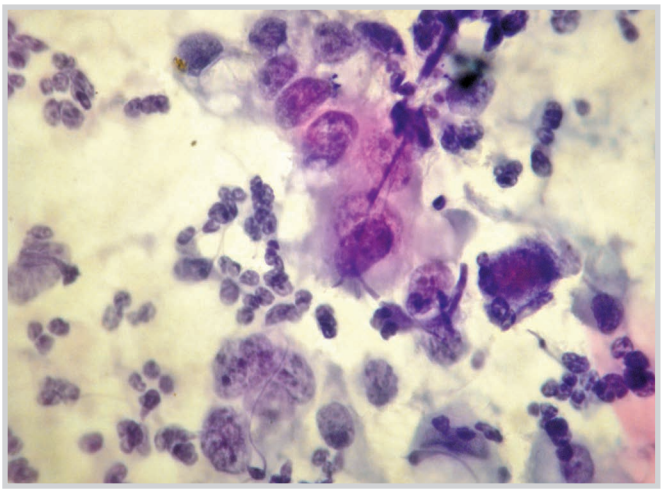


Fig. 8.40: Many discrete cells with moderate nuclear pleomorphism, coarse chromatin and large prominent nucleoli in adenocarcinoma of cervix (Papanicolaou's stain X HP)

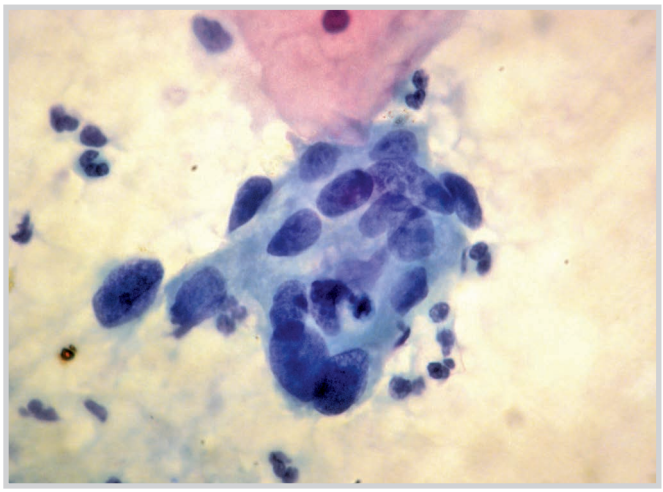


Fig. 8.38: Cells with enlarged nuclei and moderate amount of vacuolated cytoplasm in adenocarcinoma of cervix (Papanicolaou's stain X HP)

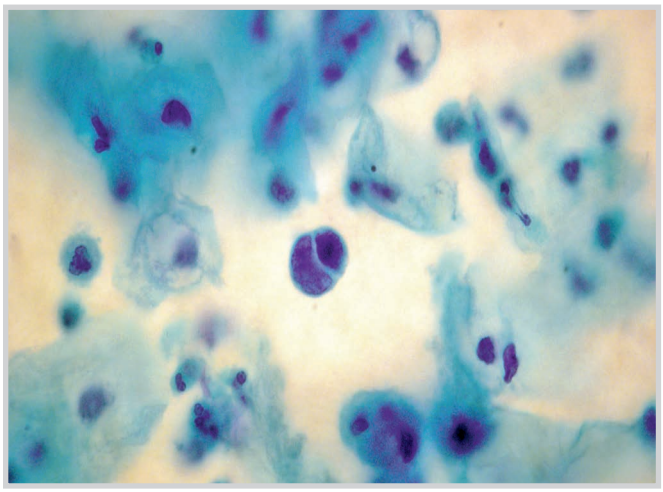


Fig. 8.41: LBC preparation showing discrete malignant cells with clear background in adenocarcinoma of cervix (Papanicolaou's stain X HP)

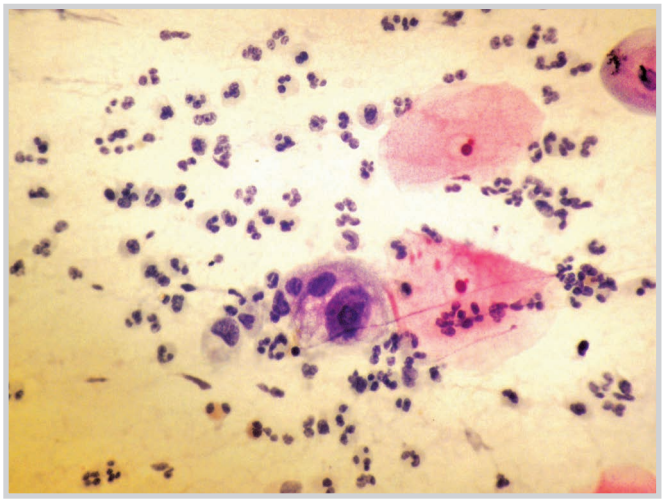


Fig. 8.39: Cells with moderate nuclear pleomorphism, coarse chromatin and large prominent nucleoli in adenocarcinoma of cervix (Papanicolaou's stain X HP)

TABLE 8.4: Differentiating features of endocervical and endometrial adenocarcinoma

Features	*Endocervical adenocarcinoma*	*Endometrial adenocarcinoma*
Age	Premenopausal, younger patient	Postmenopausal, older patient
Cellularity	Abundant	Relatively scanty
Cell arrangement	Discrete and sheets	Discrete and spherical clusters
Cell appearance	Columnar	Round to oval
Nucleus	Moderate enlargement	Relatively smaller
N/C ratio	Low	High
Histiocytes	Absent	Present

BOX 8.8 **Endometrial adenocarcinoma**

- Highly cellular smear
- Cells are present discretely or in tight clusters
- Nucleus
  - Loss of polarity
  - Large
  - Hyperchromatic
  - Pleomorphic
  - Multiple nucleoli
- Cytoplasm
  - Scanty, vacuolated
- Tumor diathesis: finely granular
- Histiocytes

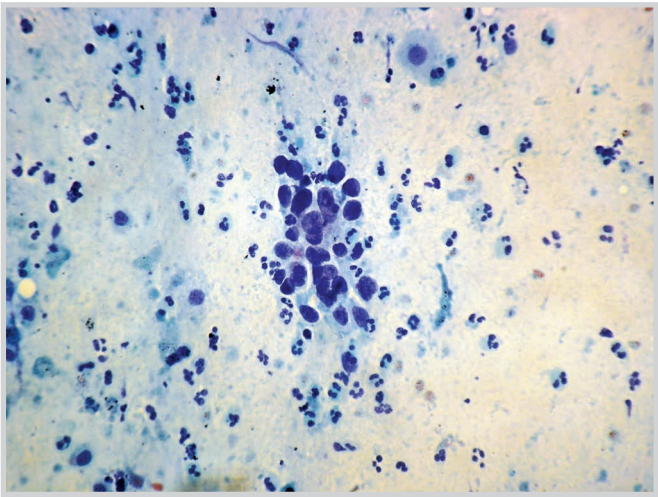


Fig. 8.42: Cervical smear showing tight clusters and discrete tumor cells in endometrial adenocarcinoma (Papanicolaou's stain X HP)

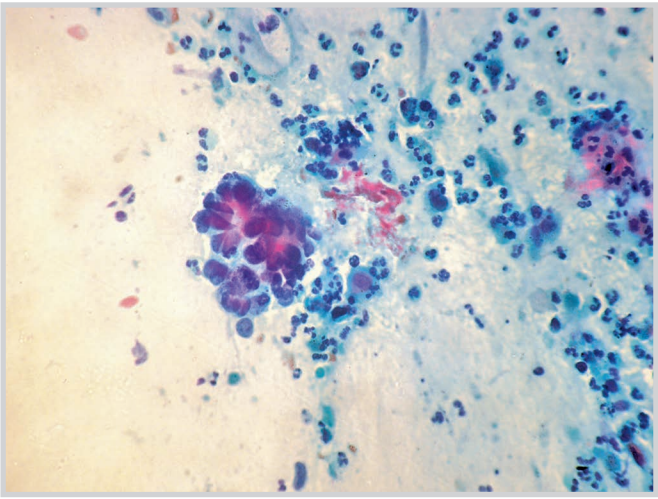


Fig. 8.43: Cluster of cells with overlapping nuclei in endometrial adenocarcinoma. The nuclei are dark and occupy whole of the cytoplasm (Papanicolaou's stain X HP)

Cytological Features of AGC, Endocervical in Origin (Box 8.9)

Smear shows discrete and small groups of cells with crowded nuclei. Cytoplasm of the cell is abundant and vacuolated with distinct cell border. Individual cells show mild nuclear enlargement, pleomorphism and mild hyperchromasia.

Cytological Features of Atypical Glandular Cell, Endocervical in Origin and Favors Neoplasia

Cervical smear shows sheets and tight clusters of cells. The margin of the cluster often shows nuclear palisading and feathering effect (**Figs 8.44** and **8.45**). Individual cells show moderate nuclear enlargement, pleomorphism and hyperchromasia. Mitotic figures may be seen frequently (**Box 8.10**).

Atypical Endometrial Cells Cytology

Smear shows small tight spherical cluster of cells. Individual cells have mildly enlarged hyperchromatic nuclei with small nucleoli and vacuolated cytoplasm (**Box 8.11**).

Management of AGCUS

- Colposcopy with endocervical sampling
- Woman with atypical endometrial should have endometrial sampling
- If no abnormality is identified, then excision biopsy is advised

BOX 8.9 **Atypical glandular cell (AGC), endocervical**

- Cells are in sheets or small overcrowded groups
- Cytoplasm: abundant, vacuolated with distinct cell border
- Nucleus
  - Mild enlargement: Three times than normal endocervical nuclei
  - Mildly pleomorphic
  - Hyperchromatic
  - Small nucleoli
- Rare mitotic figures

BOX 8.10 **Atypical glandular cell, endocervical origin, favors neoplasia**

- Sheets and hyperchromatic crowded groups of cell
- Clusters show feathering effect
- Nucleus: enlarged, hyperchromatic, pleomorphic with irregular margin
- N/C ratio increased
- Mitosis increased

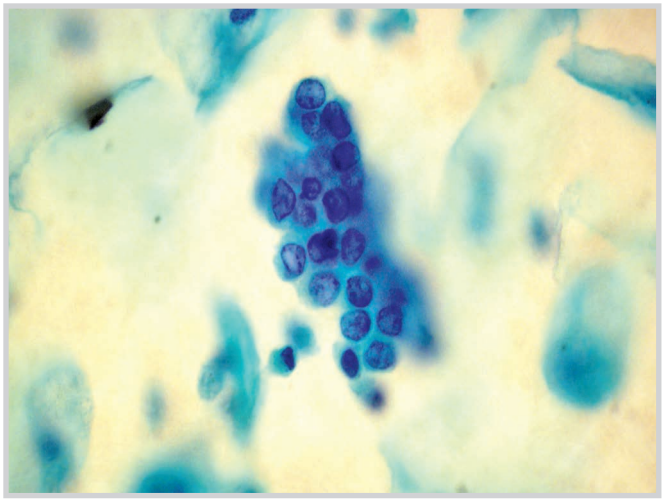


Fig. 8.44: Cluster of cells with enlarged nuclei in AGCUS (Papanicolaou's stain X HP)

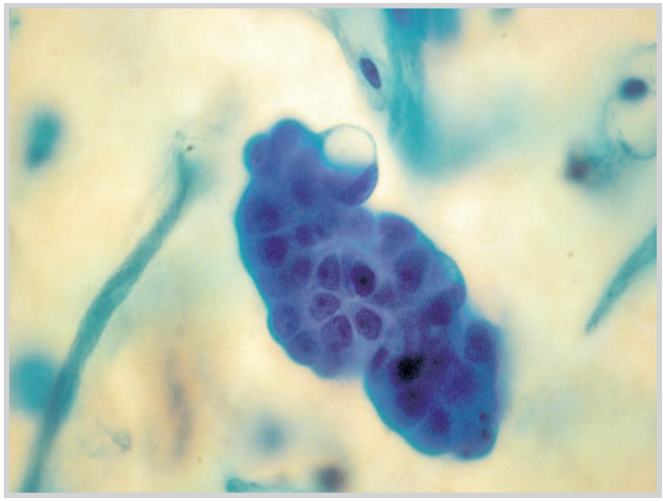


Fig. 8.45: Cluster of cells with enlarged nuclei and vacuolated cytoplasm in AGCUS (Papanicolaou's stain X HP)

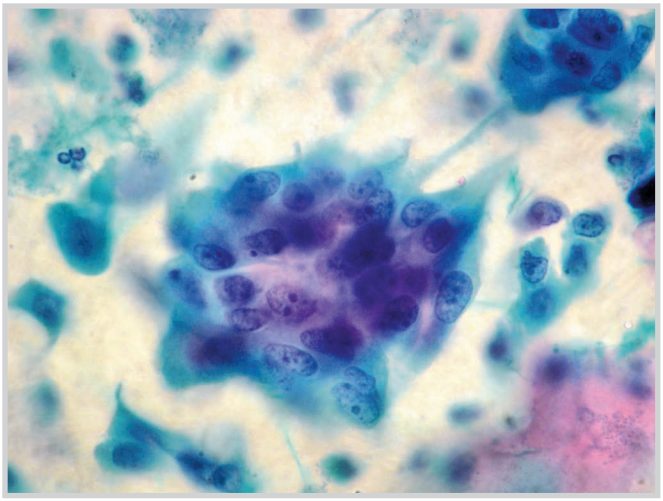


Fig. 8.46: Liquid-based cytology (LBC) smear (SurePath) of a case of adenocarcinoma of the ovary showing clusters and discrete malignant cells (Papanicolaou's stain X HP)

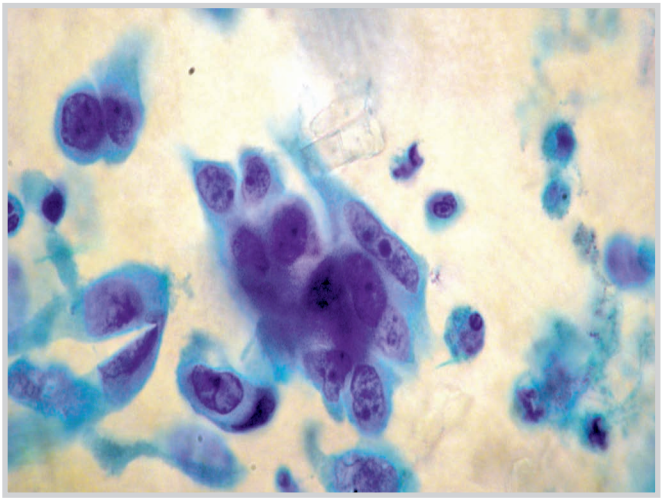


Fig. 8.47: Liquid-based cytology (LBC) smear (SurePath) of a case of adenocarcinoma of the ovary showing moderately pleomorphic nuclei with single to multiple prominent nucleoli (Papanicolaou's stain X HP)

BOX 8.11 **Atypical endometrial cells**

- Small groups of cells
- Nucleus
  - Mildly enlarged than normal endometrial cell.
  - Mildly hyperchromatic
  - Small nucleoli
- Cytoplasm: Vacuolated

- If no abnormality is identified, then repeat PAP smears at 4–6 months interval should be done until 4 consecutive samples are negative.

EXTRAUTERINE CARCINOMA

Uncommonly, cervical smear may show malignant cells from ovary or fallopian tube. The malignant cells may either come to the cervix through the transtubal and uterine passage or they may be present due to the metastasis of the tumor in cervix. The cytomorphology of the malignant cells depends on the histological features of the primary malignancy. The thorough clinical history is helpful for the exact diagnosis of metastatic carcinoma. Adenocarcinoma of the ovary usually shows clusters and discrete malignant cells in cervical smear (**Fig. 8.46**). Individual cells show moderately pleomorphic nuclei with single to multiple prominent nucleoli (**Fig. 8.47**). Unlike uterine or cervical adenocarcinoma, tumor diathesis is not seen in such cases.

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CHAPTER 9

Cervical Cancer Screening Program

Chapter Contents

INTRODUCTION

Cervical cancer is the second most common cancer in women in the world and the most common cancer in developing countries.[1] It is considered as a leading public health problem. Detection of cervical cancer on cervical cytology has helped a lot to decrease the incidence of cervical cancer in developed countries.

SCREENING PRINCIPLE

Screening is primarily used to detect the possible disease in a population who have the disease, but asymptomatic. Therefore, screening tests should have high sensitivity. According to World Health Organization (WHO), a screening program should only be implemented when its efficacy is proven; resources are sufficient to cover the entire target population; and facilities should be existing to confirm the diagnosis, follow-up the cases and treatment (**Box 9.1**).[2] A screening test should be cheap, easy to do, widely acceptable and efficient to pick up the disease.

The screening test should be done only for the diseases with considerable morbidity and mortality. It is also preferable to know the natural history of the disease for effective control of the disease in early stage. Effective screening should reduce the morbidity and mortality of the disease.

BOX 9.1 **Screening test**

- High sensitivity and reasonable specificity
- Enough resource to cover the entire population
- Facilities to have confirmation of diagnosis and treatment of the disease
- Cheap
- Easy to do
- Acceptable to the population
- Screening test should be done for disease with high morbidity and mortality

PARAMETERS TO MEASURE THE VALIDITY OF SCREENING TESTS

There are several parameters to measure the efficacy of a screening test.

Sensitivity

Sensitivity refers to the ability of the test to detect the disease that means it detects the proportion of positive result among the total number of disease positive cases.

$$\text{Sensitivity} = \frac{\text{true positive}}{\text{true positive} + \text{false negative}} \times 100\%$$

Specificity

This term means the ability to exclude the presence of disease that means it indicates the percentage of negative cases reported among the total number of negative cases.

$$\text{Specificity} = \frac{\text{true negative}}{\text{true negative} + \text{false positive}} \times 100\%$$

True positive means a genuine indication of the presence of disease and true negative means a genuine absence of the disease. Similarly, false positive means the test reported as positive but in reality the person is free of disease. False negative indicates that the test reported as negative but in reality the case is positive of the disease.

Positive Predictive Value

Positive predictive value (PPV) measures the reliability of positive tests in predicting the presence of disease. So PPV measures the proportion of real positives among all positive results reported

$$\text{Positive predictive value} = \frac{\text{true negative}}{\text{true negative} + \text{false positive}} \times 100\%$$

The sensitivity and specificity of conventional Papanicolaou (PAP) test varies widely ranging from 30% to 80% and 86% to 100%, respectively.[3] The mean sensitivity of cervical smear is 47% and the mean specificity is 95%.

TYPES OF MODALITIES FOR CERVICAL CANCER SCREENING

There are different modalities for cervical cancer screening. These are:

- Conventional cervical PAP test
- Liquid-based cytology (LBC)
- Visual inspection of cervix with acetic acid (VIA)
- Colposcopy
- Automated cervical screening techniques
- Human papillomavirus (HPV) testing
- Polar probe

Table 9.1 has compared different modalities of cervical cancer screening.

Conventional Pap Smear

Conventional Pap smear is still considered as gold standard in cervical cytology screening. It has moderate sensitivity but high specificity. Effective Pap smear screening needs well organized program, dedicated trained personnel and good laboratory support.

Liquid-based Cytology

The sample is collected in liquid media instead of directly spreading on the glass slide. Later on, the collected sample in liquid media is processed accordingly. Liquid-based cytology helps in avoiding in air drying artifact and increased adequacy of the samples. The monolayered cellular smear is easy to screen. Moreover, the residual sample in the vial can be used for further ancillary investigations, such as HPV DNA testing. The overall adequacy rate of LBC is higher than the conventional Pap smear.

TABLE 9.1: Comparison of different modalities of cervical cancer screening

Methods	*Sensitivity*	*Specificity*	*Advantages*	*Disadvantages*
Conventional PAP test	30–80%	86–100 %	Easy, cheap, effective	Needs organized approach, laboratory based, stringent quality control needed
Liquid-based cytology [4]	61–66%	82–91%	No air drying effect, adequate, clean background, multiple smears and HPV DNA test possible from residual material	Costly, trained personnel for interpretation
VIA[5]	67–79%	49–86%	Low cost, easy to do, high sensitivity	Low specificity and high false positivity, stringent quality control is needed
Colposcopy	44–77%	85–90%	High specificity	Expensive, slow, not possible to cover large population and in low resource area
HPV test[6,7]	88–100%	61–96%	Highly sensitive, objective, reproducible, robust	Costly

Abbreviations: HPV, Human papilloma virus; PAP, Papanicolaou; VIA, Visual inspection of cervix with acetic acid

However, the sensitivity and specificity of LBC is not remarkably high compared to conventional Pap test. The current evidence is not adequate enough to recommend replacing conventional Pap smear testing by LBC.[8] The cost-effectiveness of LBC needs to be assessed very carefully in developing countries.

Visual Inspection of Cervix with Acetic Acid

This is a relatively simple technique with high sensitivity and low specificity. Dilute acetic acid (3–5%) is applied to cervix using a cotton swab or a spray and then the cervix is inspected after 2 minutes. Detection of well-defined aceto-white area near transformation zone is regarded as positive for VIA. Aceto-white area in CIN is well demarcated, intensely opaque and localized to the squamocolumnar junction. However, aceto-white area can also be seen in squamous metaplasia, inflammation and repair. Aceto-white area is possibly due to a reversible coagulation of intracellular proteins following acetic acid application.

Colposcopy

It is one of the important modes of cervical cancer screening. Colposcopy helps to find out the source of abnormal cells in cytology smear. It is a binocular stereotactic microscope that produces magnified image of the cervical mucosa. The colposcopic appearance of the cervical mucosa depends mainly on the architecture of the surface epithelium, connecting tissue stroma and vasculature of the tissue. The normal cervical epithelium is translucent and gives pink color whereas the dysplastic epithelium is thickened with altered vascular pattern and therefore produces the white opaque appearance (**Fig. 9.1**). This is further accentuated by prior application of 3% acetic acid over the cervical mucosa. Colposcopic examination needs to consider the following points:

- *Surface alteration*: Any growth over the surface.
- *Color*: Alteration of normal pink color to white opaque area. This is due to thick surface epithelium and epithelial cells with increased nucleocytoplasmic ratio.
- *Vascular pattern*: There may be significant variation of vascular pattern in dysplastic epithelium. The following changes of vascular pattern are seen.
  - *Mosaicism*: Capillaries are arranged in such a way that the tissue looks like honeycomb.
  - *Punctuation*: End of the capillaries shows a red dot-like appearance.
  - *Atypical vessels*: Vessels show variable thickness and complex branching pattern.

Colposcopy cannot be used in routine screening because it is a time consuming procedure, expensive and needs a lot of experience.

Automated Screening

This is the machine based screening of cervical smears. This has been described in detail in Chapter 21.

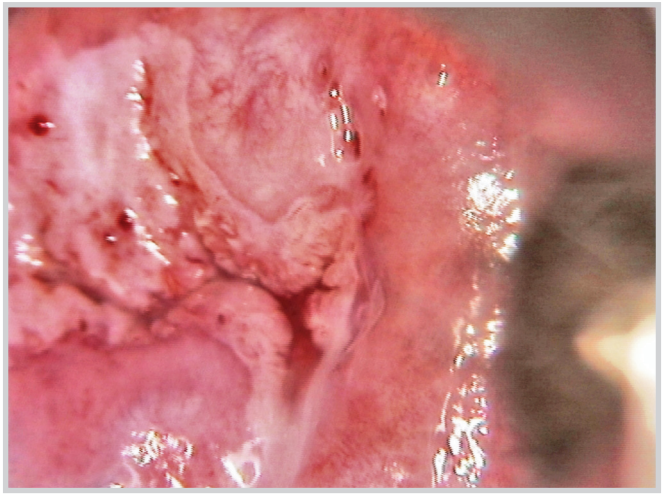

Fig. 9.1: Opaque white area on colposcopy in a case of cervical intraepithelial neoplasia
Source: Subhash Chandra Saha, Additional Professor, Department of Gynecology and Obstetrics, PGIMER, Chandigarh, India

HPV DNA Test

HPV DNA testing for high risks HPV, such as HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 59 and 68 is a potentially useful screening test. Hybrid capture technology is reliable and efficient for detection of HPV. Currently, the Hybrid capture II (HC II) assay (Digene, Silver Spring, MD) is the most useful, reliable and efficient technique for HPV DNA testing. HC II can detect 5,000 copies per sample or 1 picogram HPV DNA per sample. Non-radioactive RNA probes are used in a modified ELISA procedure to report the presence or absence of 13 strains of high risk HPV DNA.[9]

Polar Probe

It is a non-invasive electronic device used for cervical cancer screening. The polar probe is a pen shaped instrument which is connected with a computer based expert system. It measures the electrical and optical properties of the cervical tissue in vivo, and compares with the known data. The result is immediately interpreted as normal, CIN, and carcinoma. The cervical cancer screening by polar probe is still under trial.

ESSENTIAL ELEMENTS FOR SUCCESSFUL CERVICAL CANCER SCREENING

World Health Organization recommended certain essential elements of a successful cervical screening program.[1,2] Cervical screening program should be:

- The part of a national screening program and ministry of health should provide the budget
- Adequate health care system is mandatory for the implementation of a successful cervical screening program
- Target population to be screened should be well-defined

- Target population should be properly educated
- Adequate means to identify and invite the target population
- Training of the health care personnel is needed
- There should be well-defined referral center and further management of the patient whenever needed
- Proper guideline or protocol of the management
- There should be proper means to identify the failure of the program.

RECOMMENDATIONS OF CERVICAL CANCER SCREENING

Detailed guidelines of National Health Service, United Kingdom (NHS UK)[10] and American Cancer Society (ACS)[11] have been highlighted in **Table 9.2**. There is significant overlap between the recommendations of NHS UK and ACS. Both the groups recommend cervical cancer screening not before 20 years of age as there is an extremely low incidence of cervical cancer in young adult and adolescence group. As the risk of cervical cancer is very low within 3 year interval so it is recommended to have a repeat cervical smear every 3 years. An international study by IARC group showed that there is not much difference in protection if the screening frequency is 1 year rather than 3 years. Yearly screening reduces the incidence of cervical cancer by 94% whereas every three yearly reduces it by 91% and 10 yearly by 64%.[12]

PROBLEMS OF SCREENING IN DEVELOPING COUNTRIES

In developing and underdeveloped countries, cervical cancer screening program faces several hurdles. These hurdles are related with political motivation, financial strictures, proper education, etc. Important obstacles against successful cervical screening in these countries are: (1) poor health care budget on cervical screening, (2) poor financial and health care resources, (3) poorly organized health care system, (4) ill educated target population and (5) poor laboratory and other pathology facilities.[13]

False Negative Cytology

This is one of the important indicators of successful cervical cytology screening. It can be simply said that a report is a false negative when the patient has actually the disease process. Van der Graff reported false negative cytology as "cervical epithelial abnormality is diagnosed in a woman who had previously been negative but the former negative smear did not contain the abnormality or when an existing abnormality, present at that time of initial investigation, was not recognized through interpretative error or sampling error".[14] Whereas, Davey described false negative cytology as an originally negative test report that is found to have sufficient number of dysplastic or carcinoma cells on review.[15] These abnormal cells should always be verified by more than one experienced cytologists.
The false negative fraction is defined as:[16]

$$\text{False negative fraction} = \frac{\text{estimated false negative results}}{(\text{true positive results} + \text{estimated false negative results})} \times 100\%$$

The exact incidence of false negative cytology is difficult to assess due to differences in the definition of this entity. However, false negative cytology varies from 1.6% to 2.7%.[17]

The etiological causes of false negative cytology are:

- *Sampling error*: Abnormal cells are not present in the smear due to faulty sampling technique or faulty smear preparation.
- *Interpretation error*
  - Abnormal cells are not detected at all: This may be due to too little cells or cells are obscured by blood or mucus
  - Abnormal cells are wrongly interpreted as normal.

Various studies have shown that sampling error is the main source of false negative cytology.[18] The quality of the smear may be hampered by air drying effect, blood, mucus or necrotic material. Moreover, in conventional cytology, the major amount of cells is not transferred to the slide. Failure to obtain samples from transformation zone and lack of endocervical cells in the smear may be another possible cause of false negative cytology.[19]

TABLE 9.2: Comparison between National Health Service, UK and American Cancer Society

Recommendations	*NHS UK*	*ACS*
Method of screening	Conventional and LBC	Conventional and LBC
Age of first invitation for screening	England and Northern Ireland: 20 years Wales and Scotland: 25 years	21 years
Frequency of screening	Wales: every 3 yearly Scotland: every 3 yearly England: 25–49 years: every 3 years After 50: 5 yearly	21–29: every 3 year 31–65: every 5 year along with HPV test
Stop age	Wales: 64 years Scotland: 60 years England: 64 years	Up to age 65 years if the female has normal report

Abbreviations: ACS, American Cancer Society; HPV, Human papillomavirus, LBC, Liquid-based cytology; NHS UK, National Health Service, United Kingdom

Screening error may be another important cause of false negative cytology. The abnormal cells may be missed due to loss of concentration, fatigue or inadequate training.

REMEDIES TO AVOID FALSE NEGATIVE CYTOLOGY

Sampling Error

This can be avoided by: (1) adequate training for health care personnel for better sampling (2) liquid-based cytology to obtain more representative cells in clean background.

Interpretation Error

This can be avoided by

- Adequate training of the screener and cytologist
- Re-screening: Negative cytology cases may be re-screened in various ways such as:
  - 100% rapid review: To screen all negative cases rapidly within 30 seconds
  - Re-screening of 10% smear: To screen only 10% of the negative cases randomly
  - Selective re-screening: To screen only high risk individuals such as previous history of atypical cytology, prostitutes, patients with postmenopausal bleeding, etc.
  - Automated rescreening: To take the help of machine for the detection of abnormal cells and then further confirmation by a cytologist.

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CHAPTER 10

Effusion Cytology

Chapter Contents

ANATOMY AND HISTOLOGY OF BODY CAVITIES

There are three body cavities: pleural, pericardial and peritoneal (**Fig. 10.1**). These cavities and visceral organs are lined by a thin layer of mesothelial cells. Normally, these cavities are collapsed and contain only a potential space with thin lubricating fluid. The mesothelial layer just covering the visceral organ is known as visceral layer and the outer layer is known as parietal layer. Each lung in the thoracic cavity is lined by visceral pleura and the potential space is known as the pleural cavity. The outer layer of the pleura is attached to the chest wall and is known as parietal pleura. There is no anatomical connection between the two pleural spaces. Similarly, the heart is within the pericardial sac and most of the abdominal organs are within the peritoneal cavity. The peritoneal cavity is the largest serosal sac in our body and it is derived from the coelomic cavity of the embryo. It contains about scanty fluid that has lubricant and anti-inflammatory effect.

The visceral organs are covered by a single layer of small cuboidal mesothelial cells (**Fig. 10.2**). These cells rest on fibrovascular connective tissue, blood vessels and nerves.

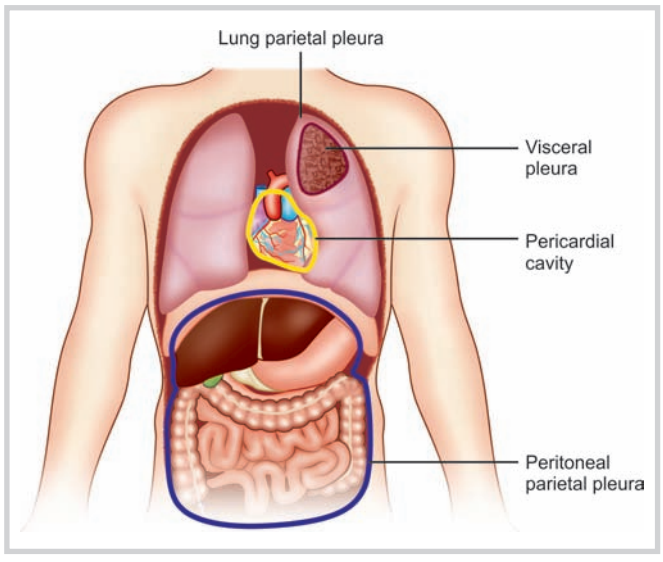


Fig. 10.1: Schematic diagram of body cavities: pericardial, pleural and peritoneal

EFFUSION

Under normal physiological condition, fluid is produced by the parietal pleura and is absorbed by capillaries and lymphatic of visceral pleura. Accumulation of excess fluid in the body cavity is labeled as "effusion". Effusion is always a pathologic process. Clinically, effusion can be divided as: transudative and exudative.

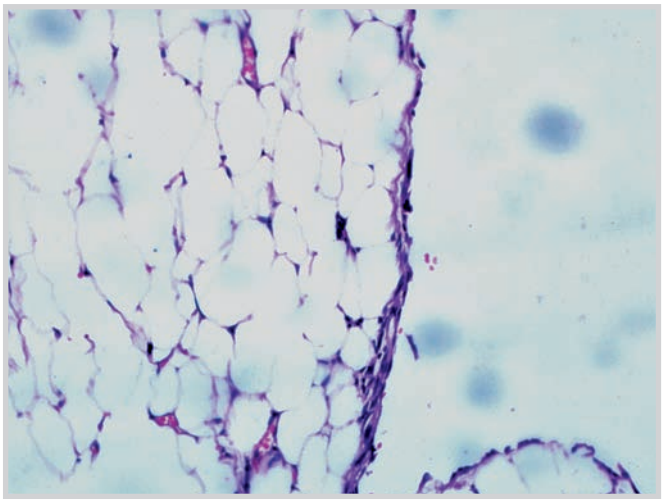


Fig. 10.2: A single layer of flat to small cuboidal mesothelial cells as lining of peritoneal surface (Hematoxylin and Eosin X MP)

TABLE 10.1: Differences between transudate and exudate

Features	*Transudate*	*Exudate*
Appearance	Clear	Turbid
Specific gravity	Less than 1.015	More than 1.015
Protein content	Less than 3 gm/100 mL	Above 3 gm/100 mL
Fibrin	Low, does not coagulate	High , coagulates if kept
Lactate dehydrogenase (LDH)	Low	High
Cells	Scanty cells	Rich in cells
Significance	Rarely contains malignant cells	Often contain malignant cells

Transudative Effusion

Transudative fluid is caused by the disturbance of hydrostatic pressure and/or osmotic pressure and it represents the ultrastructure of plasma. The wall of the blood vessels in transudative effusion is intact. The common causes of transudate are congestive cardiac failure, cirrhosis of liver and renal failure. Transudate has a low specific gravity (less than 1.015), low protein content and scanty cellularity. The cells in transudate are usually mesothelial cells and occasional lymphocytes or polymorphs.

Exudative Effusion

Exudate is caused by the damage to the wall of the blood capillaries of the mesothelial lining. This damage of the capillaries may be due to inflammation or by direct damage by malignant cells. The common causes of transudative effusions are infection, collagen vascular disease and malignancy. Comparison of transudative and exudative effusion is shown in the **Table 10.1**. Exudative effusion has a higher specific gravity (more than 1.015), high protein content (more than 3 gm/100 mL), high lactate dehydrogenase (LDH) content, high in fibrin and rich in cells.

Malignancy usually causes exudative effusion by damaging the wall of the blood vessels. However, the tumor may block the lymphatic channels by pressure effect and may cause transudative effusion.

Appearance of Effusion Sample

The following thing should be noted carefully in all effusion fluid:

Volume

Exact amount of fluid should be recorded.

Color

Color of the fluid is important. Transudative fluid is usually light straw color. Blood-tinged fluid is reddish in color. The gross presence of blood may be due to direct trauma of vessels during collection of fluid or it may indicate malignancy. Chylous fluid is milky in color that is usually noted in lymphatic obstruction. Dark brown fluid may be due to old hemorrhage. Chocolate brown fluid is noted in malignant melanoma

Consistency

Viscosity of the fluid may give important information. Fluid in mesothelioma is rich in hyaluronic acid and may have increased consistency like honey. Thick gelatinous consistency of fluid is seen particularly in pseudomyxoma peritonei.

Smell

Any unusual smell of the fluid should be noted.

SPECIMEN COLLECTION AND PROCESSING

A collection of effusion fluid from the body cavity is a relatively easy process. This is done under local anesthesia by inserting a wide bore needle into the body cavity affected by effusion. Thus the collection of effusion fluid from the thoracic cavity, pericardial cavity and peritoneal cavity is known as thoracocentesis, pericardiocentesis and paracentesis, respectively. At a time, one should not draw more than 1.5 liter of fluid from the pleural cavity as this may cause rebound phenomenon and development of pneumothorax. The effusion fluid should be collected in a clean container. We prefer to collect fluid in anticoagulant solution (9:1, fluid: ammonium oxalate). However, many laboratories prefer fresh sample without

any anticoagulant. If necessary, effusion fluid can be kept in refrigerator at 4°C for a few days. Under any circumstances fluid should not be frozen.

Processing

Centrifugation

The specimen of fluid should be shaken before the processing so that the cells are well dispersed. About 50 mL fluid is centrifuged and the supernatant is discarded. Three to four smears are made from the sediment. We usually make one air-dried smear for May-Grünwald-Giemsa (MGG) stain and three alcohol-fixed (95% ethanol) smears for Papanicolaou's stain. Extra smears are kept for further special stains, if required (**Box 10.1**).

Cell Block

It is always advisable to make a cell block from the residual sample. The residual sediment is treated with plasma followed by drops of thrombin. The sample is clotted. The clot is fixed with 10% buffered formalin for 30 minutes. The fixed clot is then wrapped in filter paper and is processed for paraffin embedding.

Advantages

- Cell block sample allows to perform special stains, immunocytochemistry, etc. because multiple slides can be made from the block
- Tissue can be kept as archival material for future use
- Morphologic comparison with histopathology section is possible
- As cell blocks enmesh all cells in clots so chances of detection of malignancy is high in the cell block.

Flow Cytometry

If necessary, the residual fluid can be used for DNA flow cytometry and dual color flow cytometry.

Liquid-based Preparation

As cells in effusion fluid is in liquid media, so it is not necessary to keep sample for liquid-based cytology (LBC). However, LBC may yield good cellularity and the cells are spread in a small area.

BOX 10.1 Processing of effusion sample

- Centrifugation: Specimen centrifuged and supernatant is discarded. Multiples smears for Papanicolaou's stain and May-Grünwald-Giemsa (MGG) stain
- Cell block: Centrifuged sample treated with plasma followed by thrombin. Clot fixed by 10% formalin and then processed in tissue processor
- Liquid-based preparation:
- Ancillary technique:
  - Sample for flow cytometry
  - Other techniques

BENIGN CELL POPULATION IN EFFUSION

The benign effusion fluid contains different types of cells consists of mesothelial cells, macrophages, lymphocytes, polymorphs, etc.

Mesothelial Cells

These are the most common cells noted in effusion (**Box 10.2**). Effusion, due to irrespective of the causes, contains mesothelial cells. These cells are shaded from the visceral or parietal lining and freely proliferate in the effusion fluid. Therefore, mesothelial cells may often show reactive changes and simulate malignant cells.

The mesothelial cells in smear are usually present discretely or as small monolayer clusters (**Figs 10.3A and B** and **10.4**). The individual cells are round to oval with moderate cytoplasm and central to eccentric nucleus. The nuclei show mild nuclear pleomorphism, smooth contour with uniformly granular chromatin and readily visible nucleoli. Mesothelial cells often show bi-nucleation, multinucleation (**Fig. 10.5**), nuclear pleomorphism and multiple prominent nucleoli. However, the nuclei of the cells do not show marked atypia and these cells often attach to the other mesothelial cells that give indication of their mesothelial origin.

The groups of mesothelial cells often show a window like space in between the cells. This is due to the long slender microvilli on the surface of the cells that has been demonstrated ultrastructurally by scanning electron microscopy.

Mesothelial cells often show bi-nucleation, multi-nucleation and marked nuclear pleomorphism. This nuclear atypia depends upon the duration and intensity of inflammation. Therefore, the interpretation of effusion cytology smear should be carefully done and overall cellular background and clinical history should be given importance (**Box 10.3**).

Long standing effusion may cause small to large vacuoles within the cytoplasm of the mesothelial cells. At times the vacuoles may be quite large and may push the nucleus to the periphery giving a signet ring-like appearance (**Fig. 10.6**). Benign mesothelial cells with signet ring appearance should be distinguished from the malignant cells with signet ring cell. Nuclear morphology is the most important distinguishing feature to differentiate these two entities (**Box 10.4**).

Differential Diagnosis

Macrophages

Mesothelial cells are difficult to distinguish from the macrophages (**Table 10.2**). Macrophages often contain phagocytosed material in their cytoplasm. Nuclei of macrophages are central in position with indistinct nucleoli.

Mesothelioma

Malignant mesothelial cells may simulate reactive mesothelial cells (**Table 10.3**). The mesothelioma cells are in large groups, with moderate nuclear pleomorphism and often show macronuclei. Clinical history of asbestos exposure, rapidly developing effusion, mass lesion near mesothelial surface etc. may be helpful in difficult situations.

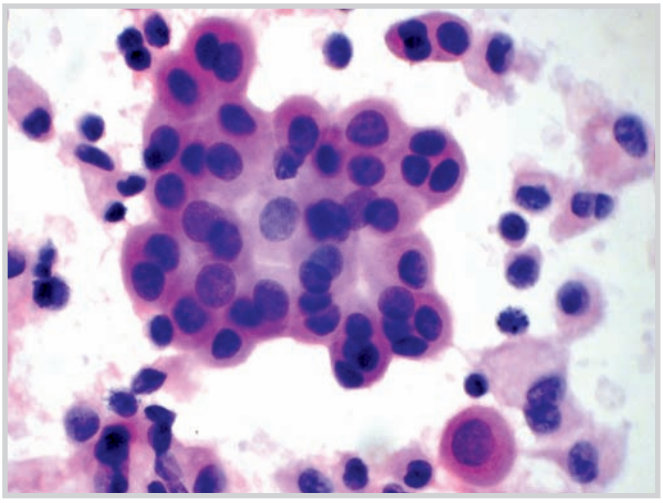


Fig. 10.3A: Clusters and discrete mesothelial cells in peritoneal effusion (Papanicolaou's stain X HP)

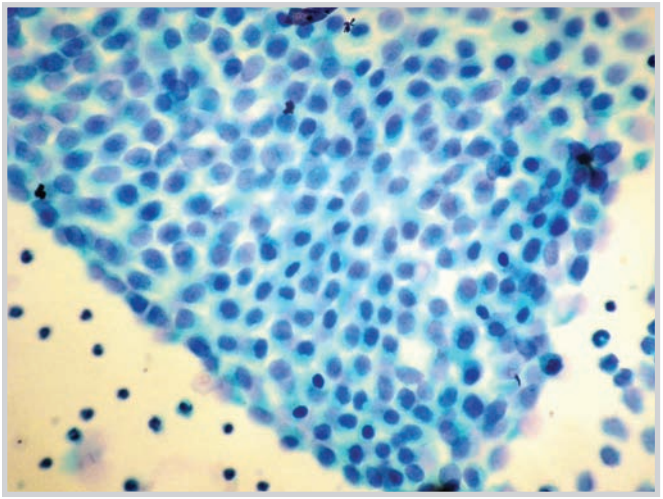


Fig. 10.4: Flat sheet of mesothelial cells with window-like gaps in between the cells (Papanicolaou's stain X MP)

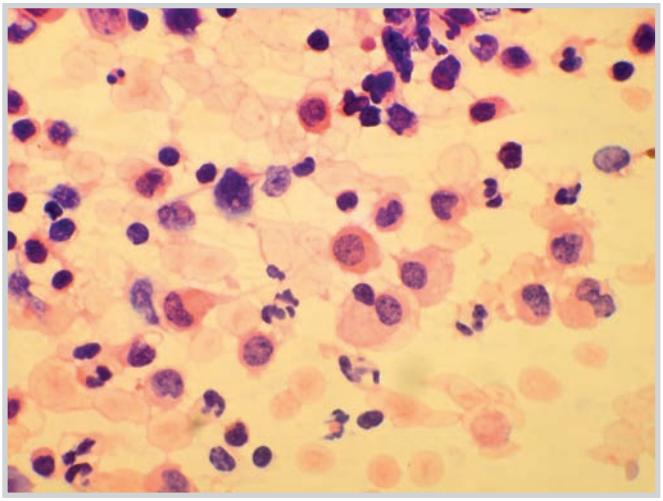


Fig. 10.3B: Discrete mesothelial cells in a benign effusion (Papanicolaou's stain X MP)

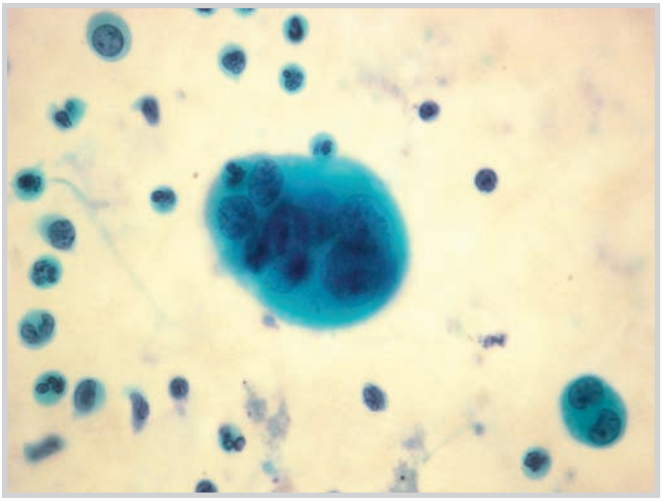


Fig. 10.5: Multinucleated giant cells in a benign effusion (Papanicolaou's stain X MP).

BOX 10.2 Mesothelial cells

- Single and small clusters
- Uniform cell population
- Window like pattern in between two adjacent cells
- Round cells with round nuclei
- Central to eccentric nuclei
- Prominent nucleoli
- Well defined cytoplasmic border
- Cells appear to be surrounded by adjacent cell
- Cytoplasmic outpouchings or blebs
- Aggregates of cell may be arranged around a central lumen
- Cytochemistry:
  - Alcian blue positive
  - Periodic Acid-Schiff (PAS) positive

Immunocytochemistry

- Positive for calretinin

BOX 10.3 Interpretation of mesothelial cells

- Do not over interpret nuclear atypia of mesothelial cells
- Appropriate importance should be given to
  - Clinical details
  - Background cellular details such as intense inflammation
- Do not interpret a compromised smear
- Two cell population usually indicates malignancy
- Intense mesothelial cell hyperplasia is seen in
  - Cirrhosis
  - Uremia
  - Pulmonary infarct
- "Atypical mesothelial cell" terminology should be avoided

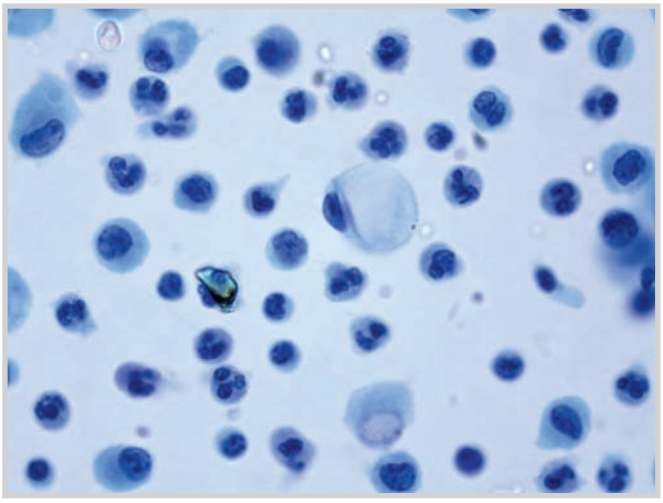


Fig. 10.6: Signet ring like-cells in a benign effusion in liquid-based cytology preparation (Papanicolaou's stain X MP)

TABLE 10.2: Comparison of mesothelial cells and macrophages

	Mesothelial cells	*Macrophages*
Cell arrangement	Cluster of cells showing window pattern	No such window pattern
Cytoplasm	Vacuolated cytoplasm	Phagocytosed material
Cytoplasmic margin	Sharp, distinguishable from background	Ill-defined and blends with the background
Nuclear position	Eccentric	Central
Nuclear shape	Round	Kidney-shaped, indented
Nucleoli	Small conspicuous	Indistinct

BOX 10.4 Signet ring-like cells in effusion

- Long standing effusion may show such cells
- Round cells with abundant vacuolated cytoplasm and peripherally pushed nuclei
- Nuclear morphology is helpful in distinguishing the character of such cells
- Other conditions where such cells may be seen:
  - Signet ring carcinoma of stomach
  - Breast carcinoma
  - Ovarian adenocarcinoma
  - Therapy related changes

TABLE 10.3: Comparison of reactive mesothelial cells and mesothelioma

Features	*Reactive mesothelial cells*	*Mesothelioma*
Cell cluster	Small-loosely adhesive	Large clusters of cells, more than 10 to 12 cells
Cell size	Small	Relatively large
Nuclear pleomorphism	Mild	Moderate
Nucleoli	Small	Large

Histiocytes (Macrophages)

Histiocytes are almost invariably present in all sorts of effusion fluid. The number of histiocytes in effusion fluid varies. In case of chronic infection, the number of macrophages or histiocytes may be increased. These cells originate in the bone marrow and come to the effusion fluid by circulation.

Histiocytes are usually present as dispersed population of cells (**Table 10.2**). However, occasionally they may form loose aggregates. In contrast to mesothelial cells they do not form any window pattern. Individual cells, are round to oval with moderate amount of vacuolated cytoplasm that often contains phagocytosed particles (**Fig. 10.7**). The cytoplasmic margin of the histiocytes is ill-defined and blends with the background. Cytoplasm may contain single large vacuole or multiple small vacuoles. Nucleus of the cell is in a central position and reniform in shape with small nucleoli and fine granular chromatin. The nucleus is usually single in number but they may be bi or multiple in number.

Other Blood Cells

Polymorphs

Scanty number of polymorphonuclear leukocytes is invariably present in effusion fluid. However, a large number of neutrophilic leukocytes are seen in purulent effusion. The causes of purulent effusion are mainly acute infection and infraction.

Lymphocytes

The presence of lymphocytes is extremely common in effusion. The predominant population of lymphocytes is seen in any chronic infection such as tuberculosis, chylous effusion and infiltration by lymphoma. Lymphocytes are polyclonal in origin, however, most of the lymphocytes are T cell type.

Eosinophils

The term eosinophilic effusion is labeled when the predominant population of cells (more than 50%) in effusion is eosinophils. Eosinophils are seen in the varieties of effusion such as allergic condition, parasitic infection, pulmonary infarct, pulmonary tuberculosis, malignancy and spontaneous pneumothorax. In certain cases, no specific cause of effusion may be found. This may be due to idiopathic eosinophilic effusion.

Mast Cells

The presence of mast cell is not rare in effusion fluid. These are mononuclear cells with blotchy purple colored metachromatic

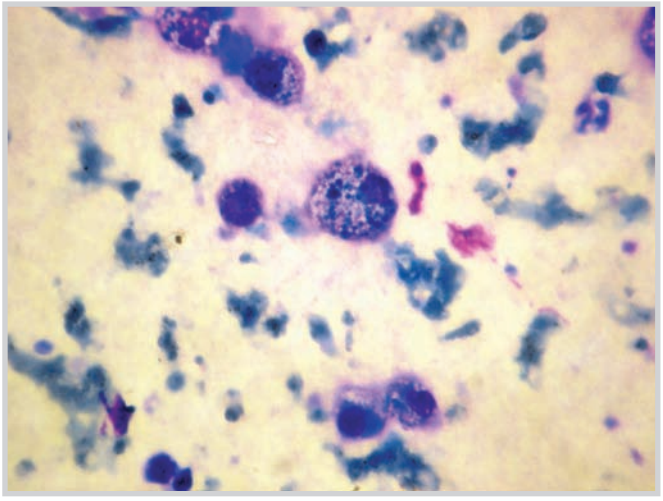


Fig. 10.7: Foamy macrophages with abundant vacuolated cytoplasm along with phagocytosed material (May-Grünwald-Giemsa X MP)

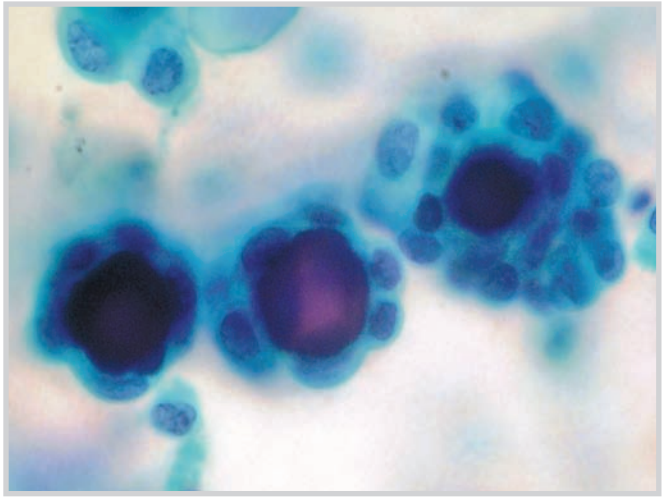


Fig. 10.8: Concentric calcified psammoma bodies in effusion smear (Papanicolaou's stain X HP)

granules in the cytoplasm in MGG stain. These cells are not recognizable in Papanicolaou's stain.

Other Cellular and Noncellular Substances

Various other cellular and noncellular substances may be present in the effusion fluid that may be helpful in diagnosis.

Psammoma Bodies

Concentric-laminated dark blue calcified material known as psammoma body may be seen in effusion fluid. Psammoma body is usually related with metastatic papillary adenocarcinoma of ovary (**Fig. 10.8**). However, the presence of psammoma body does not always indicate malignancy. It may be noted in benign effusion also.

Melanin Containing Cells

Cells with dark brown melanin containing pigment may be noted in effusion with malignant melanoma.

Hemosiderin laden crystal: Golden brown hemosiderin laden macrophages may be seen in chronic hemorrhagic effusion.

Crystals: Various types of crystal such as oxalate crystal, cholesterol crystal, Charcoat Leyden crystal etc. may be noted in effusion.

EFFUSION DUE TO NON-NEOPLASTIC CAUSES

Effusion may be caused by a large number of diseases. However, the cytological features of effusion are nonspecific and do not indicate the exact cause.

Tuberculous Effusion

In tuberculosis two things may happen (**Box 10.5**):

1. Indirect involvement of pleura: This may occur due to a hypersensitivity reaction. In this condition, effusion fluid shows predominantly lymphocytes (**Fig. 10.9A**). Occasional plasma cells, polymorphs and mesothelial cells are also noted.
2. Direct involvement of pleura: In case of direct involvement of pleural surface, effusion fluid shows a large number of mesothelial cells. Multinucleated giant cells or epithelioid cell granulomas are rarely noted (**Fig. 10.9B**).

In tuberculous effusion, predominantly T lymphocytes are seen and out of which majority is CD4+ subpopulation. Diagnosis of tuberculous effusion is not possible on the basis of cytological examination of effusion fluid. However, the presence of tuberculosis can be confirmed by demonstration of bacterial genome with the help of PCR.[1]

Rheumatoid Arthritis

Only a small population of rheumatoid arthritis patients develops pleural effusion (less than 5%). The pleural manifestation of rheumatoid arthritis generally precedes joint manifestation.

Cytological features of effusion fluid in rheumatoid arthritis are pathognomonic and diagnostic (**Box 10.6**). Smears show greenish, pink or orange color granular debris on the background in Papanicolaou's stain. This granular material is developed from the breakdown of macrophages. The material is amorphous, soft and fluffy-looking. The predominant population of cell is the macrophage. Individual cells are round to spindle-shaped. Multinucleated spindle-shaped elongated histiocytes are also seen. The nuclei of each cell may be 10–20 in number. The cells have moderate cytoplasm with elongated ends. In addition, there may be polymorphs and lymphocytes with a striking absence of mesothelial cells.

BOX 10.5 Tuberculosis

- Indirect involvement of pleura:
  - Predominantly lymphocytes
  - Occasional plasma cells, polymorphs and mesothelial cells
- Direct involvement of pleura:
  - large number of mesothelial cells

Effusion Associated with Pneumonia

Pneumonia is the inflammation of lung parenchyma. If this inflammation extends to the pleural surface, then effusion develops. The nature of the inflammatory cells in effusion depends on the nature of inflammation in the lung parenchyma. In bacterial pneumonia, the effusion cytology shows predominantly polymorphs in the early stage of pneumonia (**Fig. 10.10**). However, in course of time the effusion fluid shows lymphocytes as are seen in the lung parenchyma. In viral pneumonia, the predominant cell population is lymphocytes.

Systemic Lupus Erythematosus

About one third of patients with systemic lupus erythematosus (SLE) may present with pleural, pericardial and peritoneal effusion. The characteristic cell in SLE is lupus erythematous (LE) cell. It is probably an in vitro phenomenon. LE cell is the neutrophil or less often macrophage with an eccentric nucleus and glassy homogenous ingested cytoplasmic particle known as hematoxylin body (**Box 10.7**). The color of hematoxylin body is purple or blue in Papanicolaou's stain and magenta in MGG stain. Nucleus of the LE cell is crescent-shaped and pushed to the periphery of the cell (**Fig. 10.11**). The tart cell may also be seen in the effusion of SLE cases. These cells are neutrophils with an ingested bare nucleus of another cell. The chromatin of the dead degenerated nuclei may be noted. In addition to LE cell, the smears may also show polymorphs and occasional mesothelial cells. ♥

Congestive Heart Failure

Effusion in congestive heart failure is due to high hydrostatic pressure and the fluid is usually transudative unless secondarily infected. As the cause of effusion is very obvious, so practically there is no need of cytological examination.

Cytological feature is quite nonspecific and smears usually show scanty mesothelial cells, lymphocytes and polymorphs. In case of secondary infection, large number of polymorphs may be noted.

Cirrhosis

The predominant causes of ascities in hepatic cirrhosis are decreased intravascular osmotic pressure because of hypoalbumenimia and increased hydrostatic pressure due to portal hypertension. Smears usually show scanty mesothelial cells (**Box 10.8**). However, in case of long standing effusion large number of reactive hypertrophied mesothelial cells may be seen and may pose diagnostic difficulty. The presence of numerous polymorphs in effusion fluid gives a warning signal of spontaneous bacterial peritonitis.

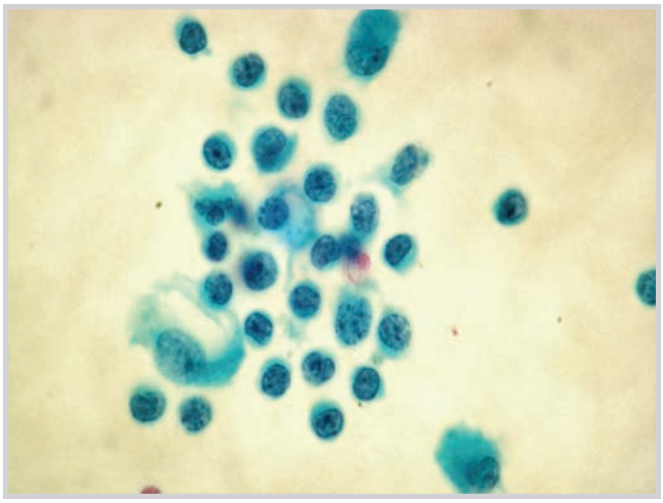


Fig. 10.9A: Effusion fluid shows predominantly lymphocytes in a case of tuberculous effusion in liquid-based cytology (SurePath) preparation (Papanicolaou's stain X MP)

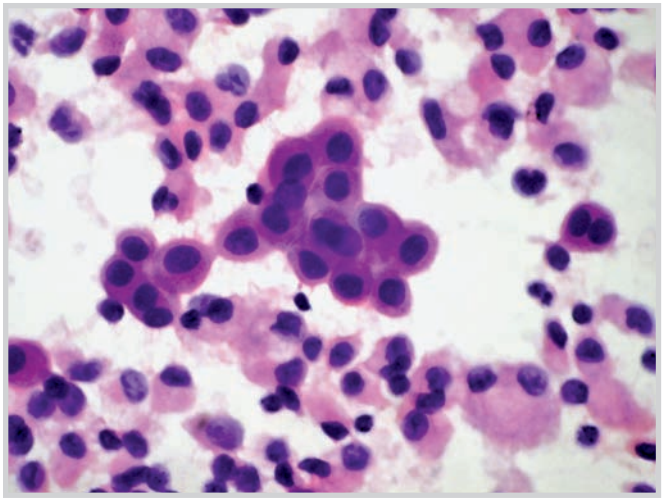


Fig. 10.9B: Effusion fluid shows predominantly mesothelial cells in a case of tuberculous effusion (Papanicolaou's stain X MP)

BOX 10.6 Rheumatoid arthritis

- Granular necrotic debris
- Oval to elongated macrophages
- Multinucleated histiocytes
- Absent mesothelial cells

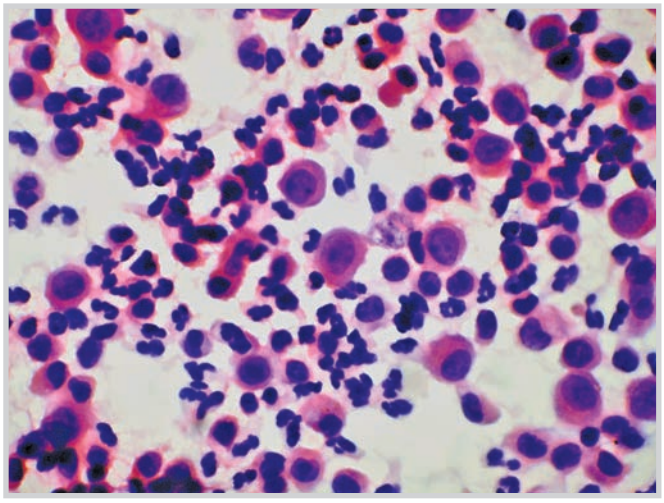


Fig. 10.10: Large number of polymorphs in early stage of pneumonia (Papanicolaou's stain X MP)

Eosinophilic Effusion

Eosinophilic pleural effusions, defined as a pleural effusion that contains at least 10% eosinophils. Common etiologic factors are listed in the **Box 10.9**.

Out of these causes, hemothorax and pneumothorax are the most common causes of eosinophilic effusion. Repeated thoracocentesis may also cause eosinophilic effusion because the procedure may introduce a small amount of air into the pleural cavity that may generate eosinophilic reaction. In one third of cases, no exact etiologic cause may be identified. Most of the eosinophilic effusions are self limiting and resolve spontaneously.

Cytology smear shows a large number of eosinophils that can be easily identified both in MGG and Papanicolaou's stain (**Fig. 10.12**). In addition, there may be polymorphs and mesothelial cells. The amount of mesothelial cells varies greatly from scanty to abundant. In case of secondary infection, the smear may contain large numbers of polymorphs.

BOX 10.7 Systemic lupus erythematosus

- Lupus erythematos cell is the characteristic cell:
  - Neutrophil or macrophages
  - Crescent-shaped nucleus pushed to periphery
  - Hematoxylin body: Homogenous, glassy, cytoplasmic material filling whole of the cell
- Tart cell: Neutrophils with ingested bare nucleus of another cell
- Scanty polymorphs and mesothelial cells

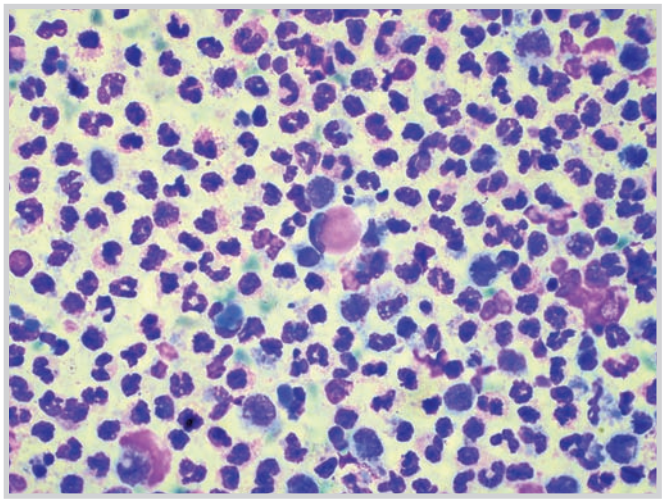


Fig. 10.11: Lupus erythematos (LE) cell in effusion fluid. Nucleus of the LE cell is crescent-shaped and pushed to the periphery of the cell (May-Grünwald-Giemsa X HP)

BOX 10.8 Cirrhosis

- Scanty reactive mesothelial cells and lymphocytes
- Long standing effusion: Abundant reactive mesothelial cells and large fragments of mesothelial cells
- Numerous polymorphs indicate spontaneous bacterial peritonitis

BOX 10.9 Etiologies of eosinophilic effusion

Pleural effusion
- Pneumothorax
- Cancer
- Postsurgical
- Traumatic
- Pulmonary embolus
- Parasitic
- Pancreatitis
- Pneumonic
- Tuberculosis
- Allergic
- Idiopathic

Pericardial
- Pulmonary eosinophilia
- Hypersensitivity reaction

Peritoneal
Allergic
Peritoneal dialysis
Parasitic

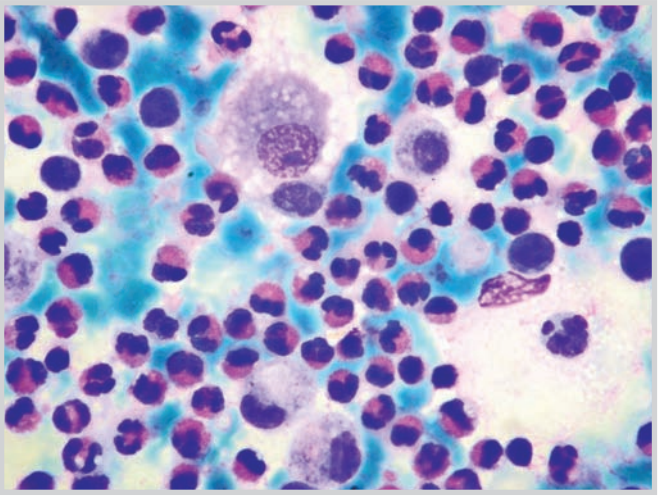


Fig. 10.12: Large number of eosinophils in a case of eosinophilic effusion (May-Grünwald-Giemsa X HP)

MALIGNANT EFFUSION

Malignant tumors often metastasize in the body cavity producing effusion. The relative frequency of pleural and peritoneal effusion in man and woman is different (**Table 10.4**).

In malignant pleural effusion, lung and breast are the most common sites in male and female patients, respectively. Similarly, in case of malignant peritoneal effusion lung and gynecologic tumors are the most common primary sites in male and female patients.

In children, non-Hodgkin's lymphomas (NHL) and hematological malignancies are the commonest malignant tumors.

In most of the cases during cytological examination of fluid, the primary malignancy is known. However, in certain cases the malignant effusion may be associated with occult primary neoplasm. Lung cancer is the commonest occult malignancy in malignant pleural effusion whereas, ovarian and pancreatic carcinomas are the commonest occult malignancy in peritoneal effusion.[2]

It is important to recognize malignant cells in effusion fluid for proper management of the patient. The primary duties of the cytologist are: (1) recognizing malignant cell and (2) detecting the exact type and origin of malignant cell.

The main challenges of the cytologist are:

- To differentiate malignant cells from the mesothelial cells
- To differentiate adenocarcinoma from mesothelioma
- To differentiate mesothelial cells from the mesothelioma.

Identification of Malignant Cells

Diagnosis of malignant cells in effusion depends on the constellation of features rather than one or two cytological features. It is important to note the overall smear pattern, background cell and cytological features of individual cells for diagnosis of malignancy in effusion fluid. In majority of the cases, morphologically recognizable malignant cells are seen in the effusion smears. Recognition of foreign or alien cells in the smear is an important step in the diagnosis of malignancy. Ancillary techniques such as cytochemistry or immunocytochemistry are rarely helpful in the diagnosis of malignancy in effusion cytology and these techniques mainly help in diagnosis of specific subtypes of malignancy. Cytologists, therefore, have to rely mainly on the morphological characteristic of the cell. **Box 10.10** highlights the essential morphological features of malignant cells in effusion.

TABLE 10.4: Common primary sites of malignancy in malignant effusion

	Male	*Female*
Pleural	Lung Hematologic Gastrointestinal tract	Breast Lung Gynecologic Gastrointestinal tract
Peritoneal	Gastrointestinal tract Miscellaneous Lung	Gynecologic Breast Lung

BOX 10.10 Cytological features of malignant cells in effusion

Cell arrangement
- Cannon ball-like tight three-dimensional cell clusters
- Papillary cluster
- Single dissociated cells in small cell carcinoma of lung
- Small rows of cells in lobular carcinoma of breast

Cytoplasm
- Single to multiple vacuoles in cytoplasm
- Keratin: In squamous cell carcinoma
- Mucus: Adenocarcinoma
- Melanin: Melanoma

Nucleus
- High N/C ratio
- Large nucleus
- Pleomorphism: Mild to moderate
- Irregular nuclear margin
- Single to multiple prominent nucleoli
- Coarse chromatin

Mitosis: Atypical tripolar mitosis

Other features:
- Cell cannibalism
- Psammoma bodies

Cytology

- *Cell configuration:* The malignant cells in the metastatic effusion fluid are recognizable on light microscopy. In fact, identification of the distinct different population of cell other than mesothelial cell is very helpful (**Fig.10.13A**). Individual malignant cells are usually round to oval. Bizarre cells are also seen. Occasionally, the cells are spindle-shaped particularly in case of sarcoma.
- *Cytoplasm:* Cytoplasm of the cells in case of adenocarcinoma often shows large single to multiple vacuoles. The vacuoles often

displace the nucleus towards the periphery giving rise to the signet ring appearance. These cells must be distinguished from the signet ring type of macrophages. Nuclei of the macrophages are small, reniform with inconspicuous nucleoli. At times, it is very difficult to assess the exact nature of these cells.

Cytoplasmic Product

Mucin: Mucin in the cytoplasm produces clear vacuoles and special stain such as mucicarmine is needed to demonstrate mucin. As benign cells rarely produce mucin so the demonstration of mucin by mucicarmine indicates malignancy.

Melanin: The presence of dark brown melanin granules in the cytoplasm of cells in malignant melanoma is characteristics. This can be confirmed by hydroxymethylbutyrate 45 (HMB 45) immunostain in smear or cell block.

Keratin: The presence of keratin within the cytoplasm is indicated by orange color in Papanicolaou's stain.

Nucleus

Nucleocytoplasmic ratio: In malignant cell; the N/C ratio is almost always high (**Fig. 10.13B**).

Nuclear chromatin: Nucleus usually shows vesicular chromatin. Cells also show irregular clumped chromatin.

Nuclear margin irregularity: The margin of the malignant cells shows an irregular outline. It is often thickened focally (**Fig. 10.13C**).

Nucleoli: The presence of multiple nucleoli of variable shape and size (nucleolar pleomorphism) is one of the important characteristics of malignant cells in effusion (**Fig. 10.13D**).

Mitosis: Qualitative abnormality of mitosis is more important rather than simply increased mitotic figures in the smear. The abnormal mitotic figures show tripolar or multipolar mitotic figures.

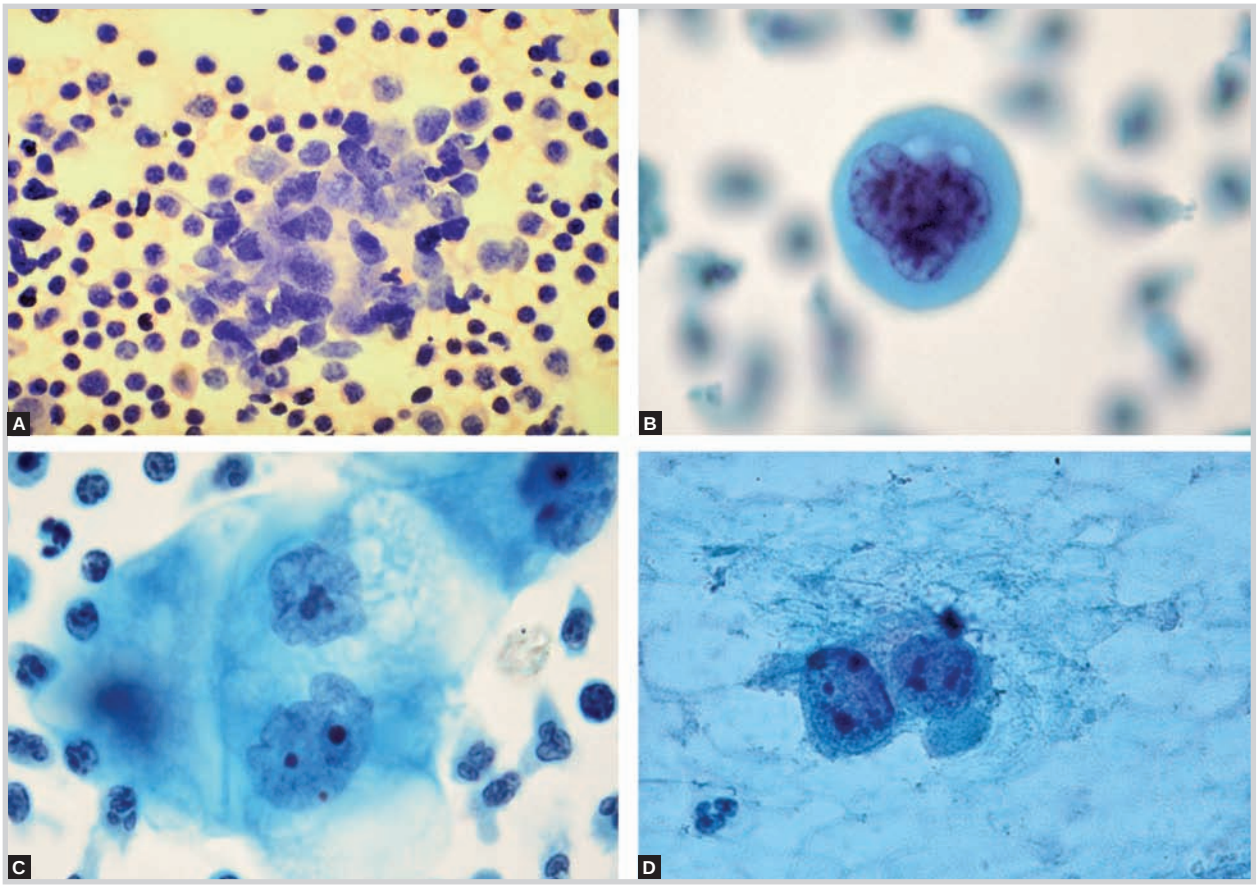
Cell cannibalism: We have noted that the increased number of cell cannibalism is one of the reliable features of malignant cell in effusion cytology smear.

Psammoma bodies: Concentric calcified bodies are often seen in malignant effusion due to metastatic serous adenocarcinoma of ovary, papillary thyroid carcinoma and carcinomatous mesothelioma.

Metastatic Tumors

Adenocarcinomas

This is the commonest malignancies in the effusion fluid. The most common primary sites are ovary, gastrointestinal tract, lung



Figs 10.13A to D: (A) Distinct two cell population is evident in metastatic carcinoma in effusion (Papanicolaou's stain X MP); (B) Malignant cell with high nucleocytoplasmic ratio (Papanicolaou's stain X OI); (C) The margin of the malignant cells show irregular outline in LBC preparation (SurePath) (Papanicolaou's stain X OI); (D) Multiple nucleoli of variable shape and size in malignant cells in effusion in liquid-based cytology preparation (SurePath) (Papanicolaou's stain X OI)

and breast. In case of malignant effusion with adenocarcinoma, the morphologically distinct foreign cell populations along with mesothelial cells are seen. Depending on the primary location of the tumor, there may be some amount of variation in morphology of the cells, however, the basic characteristics of the malignant cells are almost same. The cells are usually in a tight spherical ball-like clusters (**Fig. 10.14**). The nuclei of the cells shows overlapping and crowding. There may be hollow sphere of cells. On smear preparation, the empty space is seen in the central part of the cluster and two different layers of the cells are also identified (**Fig. 10.15**). In cell block section, the hollow sphere is seen as a central clear space with surrounding cells resembling glandular appearance. The cells may be present in single layers admixed with many mesothelial cells. Discrete recognizable malignant cells are also seen (**Fig. 10.16**). Cell cannibalism is frequently noted in malignant effusion (**Fig. 10.17**). There may be frequent bi and multinucleation. The individual cells are relatively large with moderately pleomorphic nuclei and prominent nucleoli (**Fig. 10.18**). The cytoplasm of the cells shows single to multiple large vacuoles. The vacuoles often push the nucleus to the periphery of the cell. Vacuoles may not contain mucin. However, they often contain glycogen and are Periodic Acid Schiff's (PAS) positive.

There may be predominant discrete cells in gastric and breast carcinoma. The individual cells are round to oval with eccentric nuclei containing large vacuoles giving a signet ring like appearance.

Differential Diagnosis

1. *Reactive mesothelial cells versus adenocarcinoma:* It is a great challenge to the cytopathologist to differentiate reactive mesothelial cells from adenocarcinoma. At times the morphology of reactive mesothelial cells may simulate the cells of adenocarcinoma (**Table 10.5**). The important distinguishing points between reactive mesothelial cells and adenocarcinoma are (1) ball-like three-dimensional clusters of cells, (2) increased N/C ratio, (3) large pleomorphic nucleoli and (4) irregular nuclear margin. In case of doubt, a panel of immunocytochemistry or other ancillary techniques should be applied.
2. *Adenocarcinoma versus mesothelioma:* Another challenge to the cytologist is to differentiate adenocarcinoma from mesothelioma. Cells from both mesothelioma and adenocarcinoma may show almost similar morphology. In this situation, the cytologist should depend on (1) clinical history, (2) radiological features and (3) ancillary tests such as immunocytochemistry.

Characteristic Features of Different Metastatic Carcinoma

Breast carcinoma **(Box 10.11):** Metastatic breast carcinoma is the commonest tumor in pleural effusion of a female patient. The cytology features suggestive of breast carcinoma are multiple

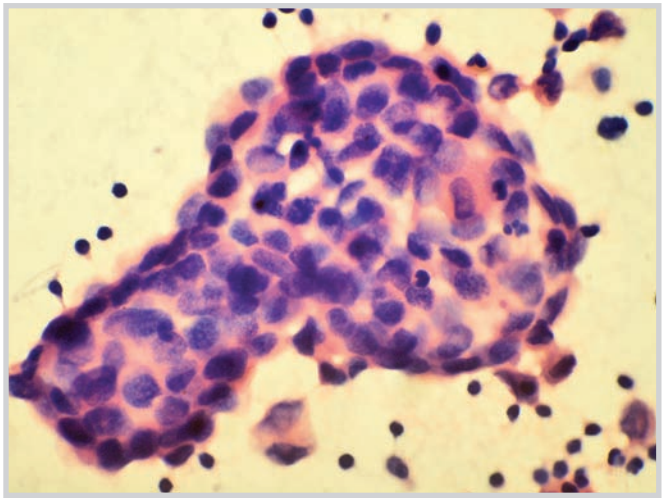


Fig. 10.15: The empty space is seen in the central part of the cluster in metastatic carcinoma in effusion (Papanicolaou's stain X HP)

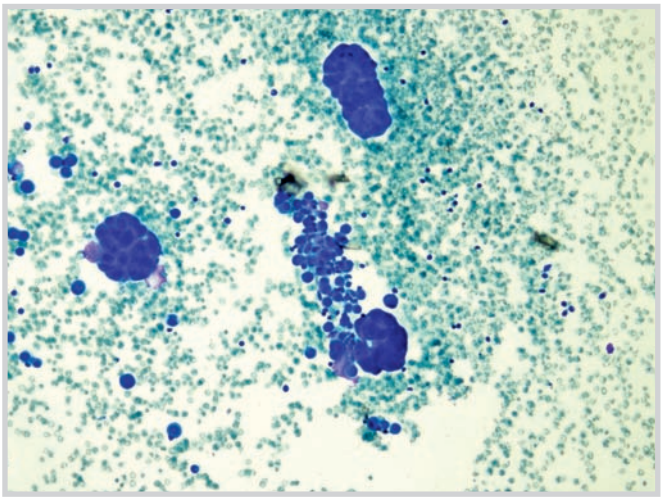


Fig. 10.14: Tight spherical ball-like clusters of malignant cells in effusion (MGG stain X MP)

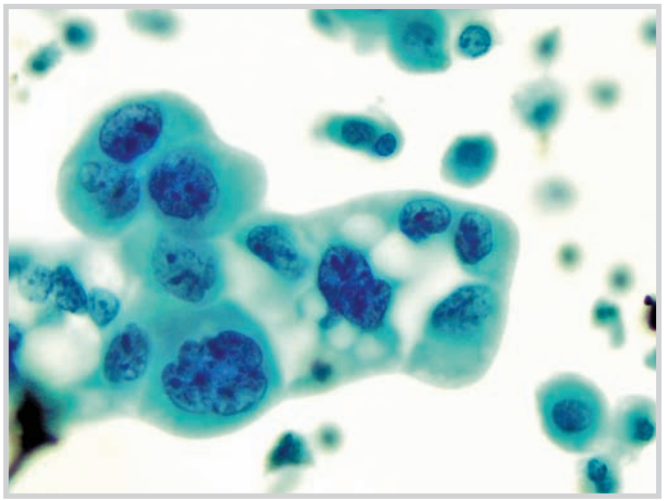


Fig. 10.16: Discrete moderately pleomorphic malignant cells in metastatic carcinoma in effusion in Surepath preparation (Papanicolaou's stain X HP)

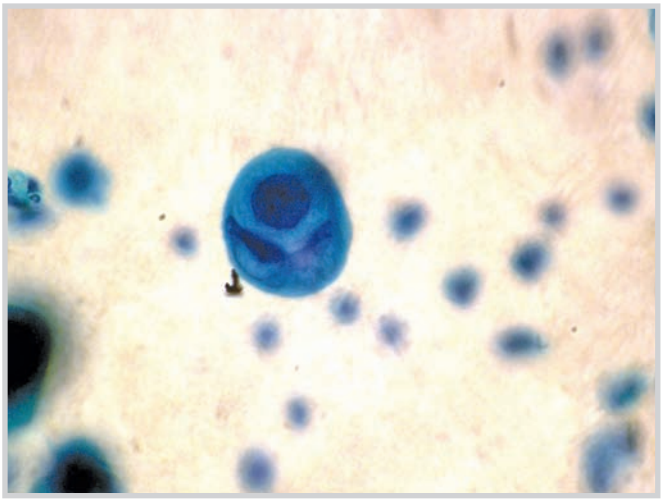


Fig. 10.17: Cell cannibalism in malignant effusion (Papanicolaou's stain X HP)

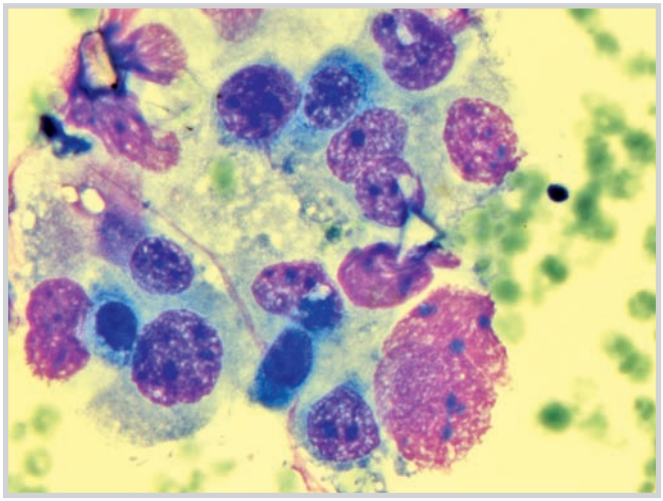


Fig. 10.18: Discrete malignant cells with vacuolated cytoplasm and large prominent nucleoli (MGG stain x OI)

TABLE 10.5: Differentiating points between reactive mesothelial cells and adenocarcinoma

Cytological features	*Reactive mesothelial cells*	*Adenocarcinoma*
Ball-like spherical clusters	Absent	Present
Two cell population	Absent	Present
Cell with high N/C ratio	Absent	Present
Large Pleomorphic nucleoli	Absent	Present
Irregular nuclear margin	Absent	Present
Sharply demarcated cytoplasmic margin	Absent	Present
Cytochemistry Mucicarmine PAS	 Negative Positive	 Positive Positive

Abbreviations: PAS—Periodic Acid Schiff's

cannon balls-like hollow spheres, acini, chain-like arrangement of the cells and intracytoplasmic lumina. The nuclei are often rectangular in shape without any molding. The cells of infiltrating duct carcinoma are usually larger and show nuclear enlargement and pleomorphism. These cells are easily recognizable. However, the cells of lobular carcinoma are smaller cells with minimal nuclear pleomorphism. These cells are often arranged as small rows simulating Indian file-like arrangement (**Fig. 10.19**). Therefore, these cells are difficult to identify in effusion.

Colonic carcinoma: Cells of colorectal carcinoma may show acini like arrangement. Individual cells are elongated columnar like with hyperchromatic nuclei and prominent nucleoli.

Gastric carcinoma: Cytomorphology of metastatic gastric carcinoma depends on whether the primary tumor is intestinal or gastric type. In gastric type of cancer, the effusion smears show dispersed cells. Individual cells are large with peripherally pushed eccentric nuclei and large vacuolated cytoplasm (**Figs 10.20A to D**). Vacuoles contain mucin and this is demonstrable by mucicarmine stain. In intestinal type, the effusion smear shows large pleomorphic cells with hyperchromatic nuclei.[3]

Ovarian carcinoma: Metastatic ovarian cancer usually presents with ascities. Isolated pleural effusion is not seen in ovarian carcinoma. The cells are usually present as large three, dimensional balls like clusters (**Fig. 10.21**). Individual cells show large vacuoles that displaced the nucleus towards periphery of the cell. The nuclei show large prominent nucleoli (**Figs 10.22** and **10.23**). Psammoma bodies are seen in serous ovarian carcinomas.

Lung carcinoma: Lung carcinoma is commonly noted metastatic carcinoma in pleural effusion fluid. The cytomorphology of metastatic lung carcinoma depends mainly on tumor type. In case of moderately differentiated bronchogenic adenocarcinoma, the cells are usually arranged in tight three-dimensional clusters. Individual cells show moderately pleomorphic nuclei with prominent nucleoli. Therefore, the cells do not show any distinguishing cytological features. In bronchioloalveolar carcinoma, the smears show uniform population of monomorphic cells with vacuolated cytoplasm.

Renal cell carcinoma: Metastatic renal cell carcinoma usually shows tight papillary clusters or acinar arrangement of cells. The individual cells are large with granular or clear cytoplasm with hyperchromatic nucleus and prominent nucleoli. The vacuoles in the cytoplasm contain glycogen or lipid (**Fig. 10.24**).

Squamous Cell Carcinoma

Squamous cell carcinoma (**Box 10.12**) uncommonly metastasizes in the serous cavities. In most of the cases, the primary sites are

BOX 10.11 **Breast carcinoma**

- Hollow spheres (cannonballs)
- Small chains or Indian file-like arrangement
- Rectangular-shaped nuclei
- Intracytoplasmic lumina

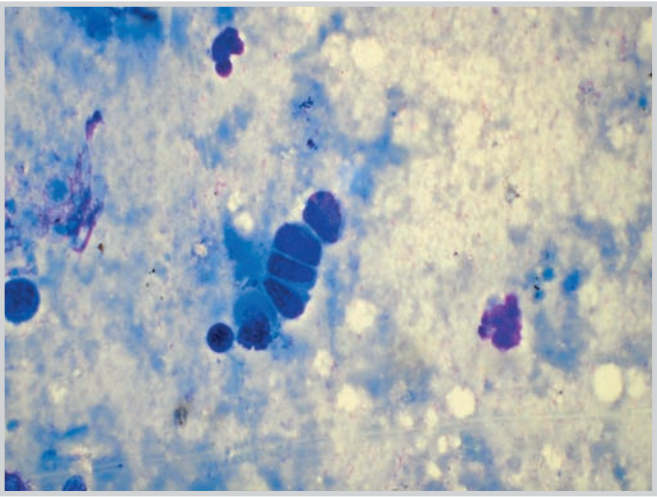


Fig. 10.19: Small rows of cell simulating Indian file-like arrangement in a case of lobular carcinoma (May Grunwald Gie msa X HP)

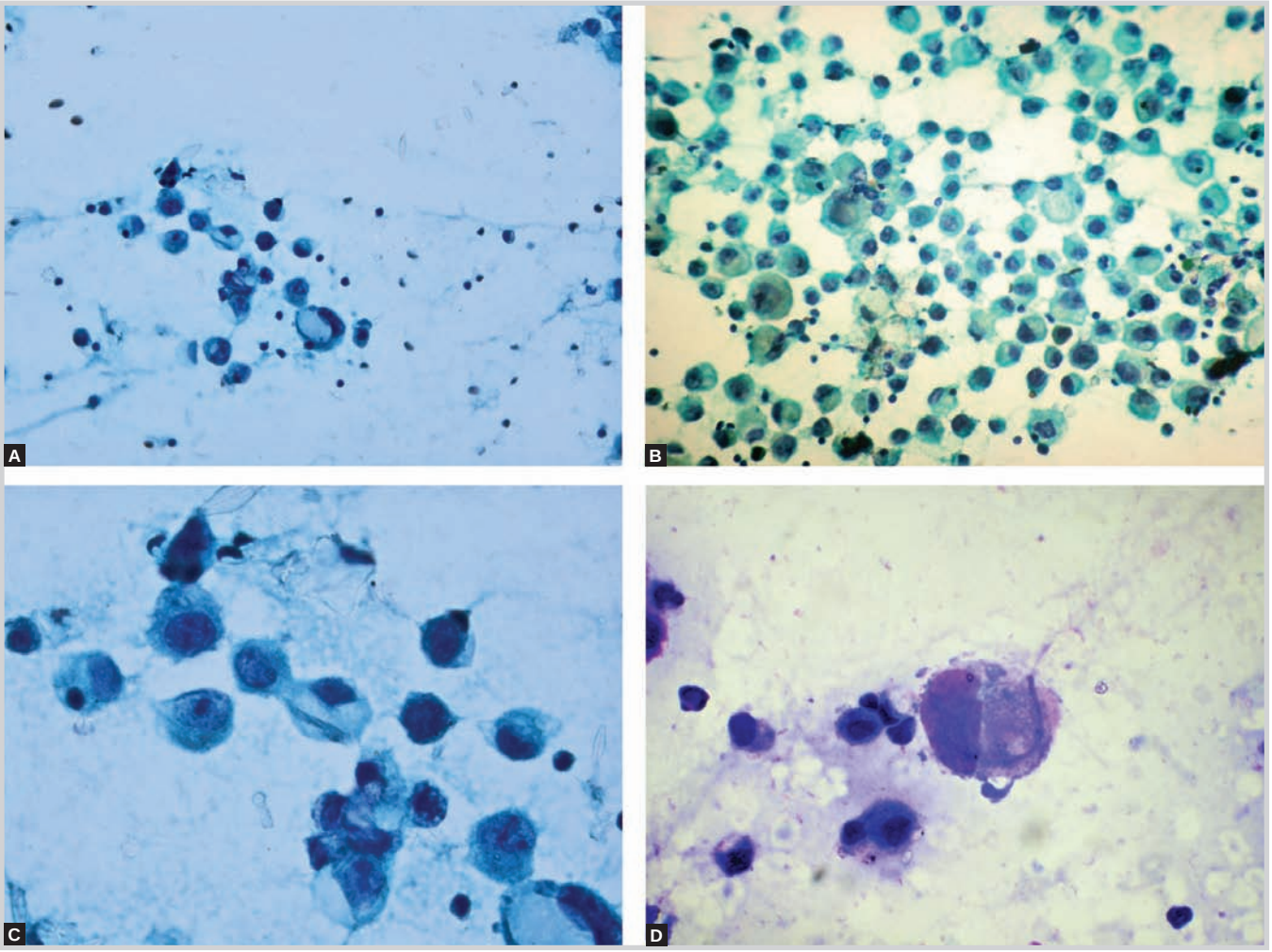
kMionown at the time of diagnosis. The common primary sites are lung, larynx and cervix.

Cytology: The cells are usually arranged as isolated discrete or small clusters. Individual cells are oval to polyhedral and occasionally spindle-shaped. Cytoplasm is orangeophilic and dense. The nucleus is hyperchromatic with inconspicuous nucleoli. Occasional aneucleated squames may also be noted. Occasional cells may show cytoplasmic vacuoles which should not be interpreted as evidence of glandular differentiation.

Small Cell Carcinoma (Box 10.13)

Metastatic small cell carcinoma is commonly derived from the lung.

Cytology: The cells are usually arranged discretely or in small chain. The individual cells are small and just two to three times larger than the lymphocytes. The cells show scanty cytoplasm and small hyperchromatic nuclei with granular chromatin and inconspicuous nucleoli. The nucleus may be fitted with each other giving angulated appearance (**Figs 10.25** and **10.26**).



Figs 10.20A to D: (A) Discrete cells with signet ring appearance in a signet ring carcinoma (Papanicolaou's stain X LP); (B) Abundant discrete signet ring cells admixed with mature lymphocytes in a case of gastric carcinoma (Papanicolaou's stain X MP); (C) Cells with abundant cytoplasm and peripherally pushed nuclei (Papanicolaou's stain X HP); (D) High powered view of a single signet ring cell (May-Grünwald-Giemsa X OI)

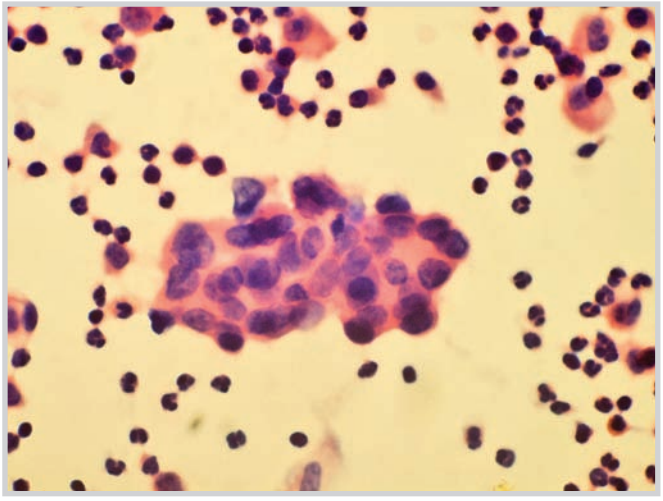


Fig. 10.21: Large three-dimensional ball-like cluster in an ovarian carcinoma (Papanicolaou's stain X HP)

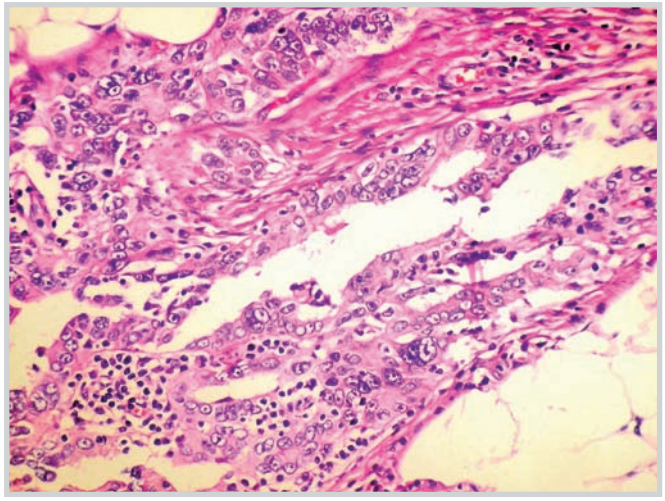


Fig. 10.23: Histopathology of the omentum shows metastatic adenocarcinoma from ovary (Hematoxylin and Eosin X MP)

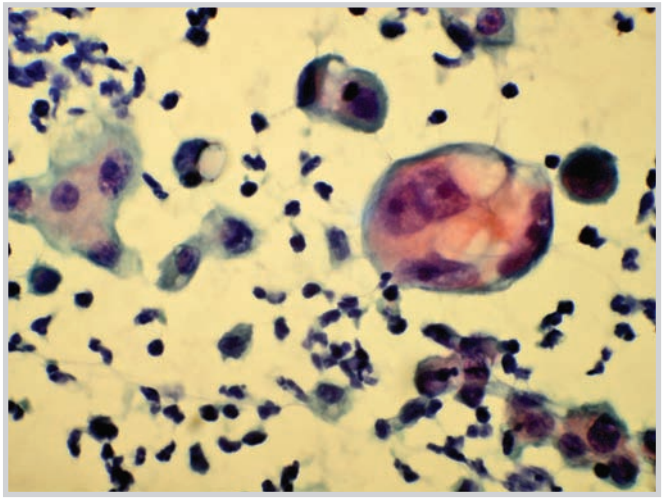


Fig. 10.22: Cells with abundant vacuolated cytoplasm with peripherally pushed nuclei (Papanicolaou's stain X HP)

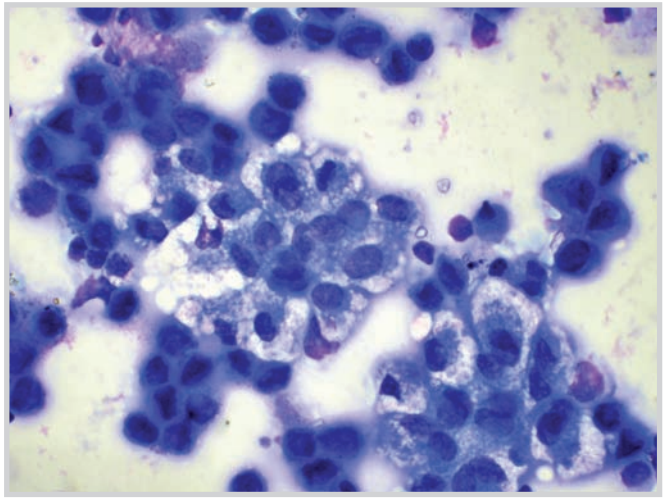


Fig. 10.24: Malignant cells with vacuolated cytoplasm in a case of metastatic renal cell carcinoma (May-Grünwald-Giemsa XHP)

Melanoma (Box 10.14)

Metastatic malignant melanoma is uncommon. It is usually associated with cutaneous or ocular melanoma. Effusion may be present without a known primary tumor or many years after the complete therapy of the primary malignancy.

The cells are usually discrete and admixed with reactive mesothelial cells. Individual cells are round to oval with moderate to severe pleomorphism**.** The cytoplasm of the cell shows single to multiple vacuoles of variable sizes. Brownish melanin pigmentation may be noted in the cytoplasm. Nuclei of the cells are central to eccentric in position with prominent large nucleoli. Intranuclear cytoplasmic inclusions are often noted.[4]

BOX 10.12 **Squamous cell carcinoma**

- Rarely metastasize in effusion fluid
- Small clusters or discrete cell population
- Oval cells in non-keratinizing squamous cell carcinoma
- Polyhedral and occasional spindle cells in keratinizing squamous cell carcinoma
- Dense and orangeophilic cytoplasm
- Hyperchromatic nucleus with inconspicuous nucleoli
- Cytoplasmic vacuole usually indicates degenerative changes

Diagnostic difficulties: Cells of amelnotic malignant melanoma may not show pigmentation. In addition, the absence of significant nuclear enlargement or pleomorphism may simulate melanoma cells as reactive mesothelial cells.[5] Immunocytochemistry particularly HMB 45 and S-100 protein may be helpful in such cases for establishing the diagnosis of malignant melanoma.

Sarcomas

Metastatic sarcoma to the serosal cavity is uncommon. However, a range of sarcomas consisting of osteogenic sarcoma, malignant fibrous histiocytoma, leiomyosarcoma, liposarcoma, synovial sarcoma and chondrosarcoma have been reported in effusion cytology.[6] Smears usually show scanty discrete malignant cells. The cytomorphology depends mostly on the exact diagnosis of the primary sarcoma. However, the cells are usually large with indistinct cell border. Cells frequently show multinucleation with bizarre nuclei (**Fig. 10.27**). Spindle-shaped fusiform or oval shaped cells have also been described.

BOX 10.13 Small cell carcinoma

- Cells in small clusters, discrete and small chain
- Small cells twice the diameter of the lymphocyte
- Scanty cytoplasm
- Nuclear molding or angulations
- Granular chromatin and inconspicuous nucleoli

BOX 10.14 Malignant melanoma

- Uncommon, may present without any history of primary melanoma
- Discrete cells
- Variable sized cytoplasmic vacuoles
- Cytoplasmic brown melanin pigmentation
- Large prominent nucleoli
- Intracytoplasmic Intranuclear cytoplasmic inclusions
- Immunocytochemistry: Positive for HMB 45, S-100, Melan A

Abbreviations: HMB 45—human melanoma black 45

Pseudomyxoma Peritonei

Pseudomyxoma Peritonei (PMP) (**Box 10.15**) is a poorly understood low grade neoplastic condition characterized by mucinous ascities and mucinous implants diffusely involving the peritoneal surfaces.[7] The most common sources of the primary tumor are mucinous adenocarcinoma of ovary, mucocele of the appendix and gastric carcinoma.

The peritoneal fluid in such cases is thick and gelatinous and difficult to spread on the slide. On MGG stain, the material looks like purple and Papanicolaou's staining shows orange to red color. The cells are scanty and are usually discrete or in small clusters. The tumor cells are round to columnar in shape with bland nuclei and clear cytoplasm (**Figs 10.28** and **10.29**).

Ronnett BM et al.[7] classified PMP as disseminated peritoneal adenomucinosis (DPAM) and peritoneal mucinous carcinomatosis (PMCA). DPAM is characterized by superficial peritoneal implants composed of abundant extracellular mucin containing scanty simple to focally proliferative mucinous epithelium with little cytological atypia or mitotic activity. DPAM has a benign and indolent clinical course. PMCA is characterized by invasive peritoneal lesions with more abundant mucinous epithelium with the architectural and cytological features of carcinoma. The prognosis of PMCA is worse than that of DPAM. This above classification is histological and has not been applied to cytology till now.

Hematological Malignancies

Serous effusion is commonly seen in lymphomas and usually the patient presents with a known history of lymphoma. About 10

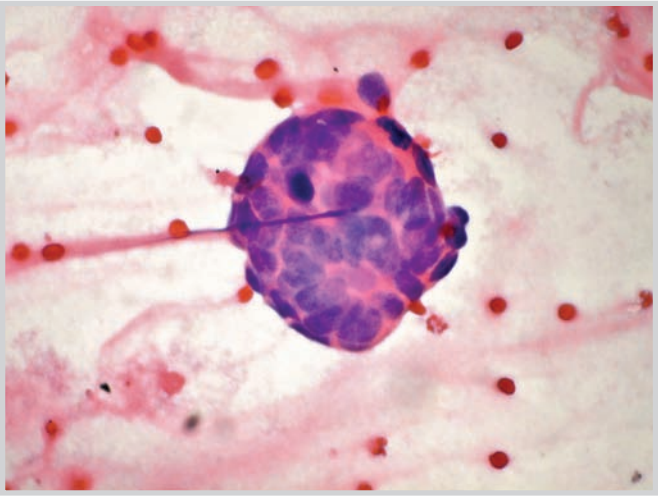


Fig. 10.25: Clusters of small cells in a small cell carcinoma in effusion (Papanicolaou's stain X HP)

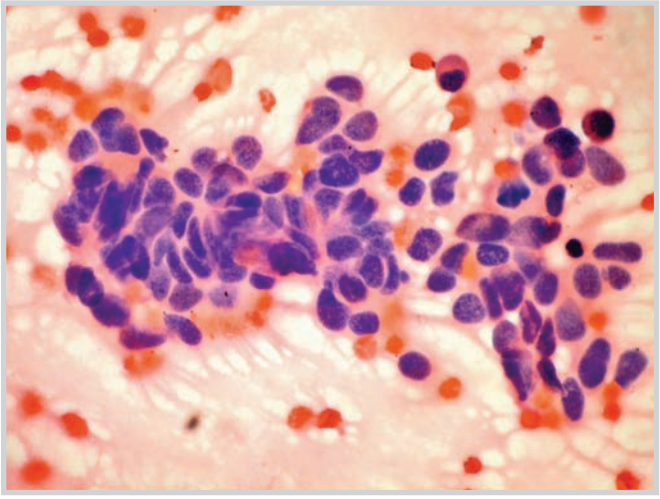


Fig. 10.26: Small cells with molded nuclei cells in a small cell carcinoma in effusion (Papanicolaou's stain X HP)

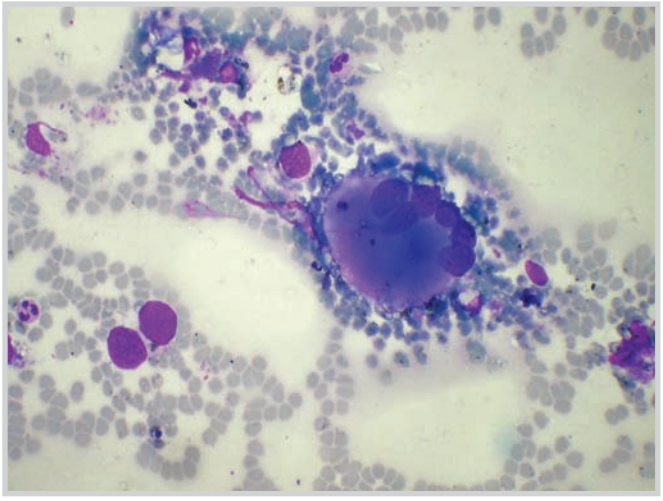


Fig. 10.27: Multinucleated bizarre cells in a sarcoma in effusion (May-Grünwald-Giemsa XHP)

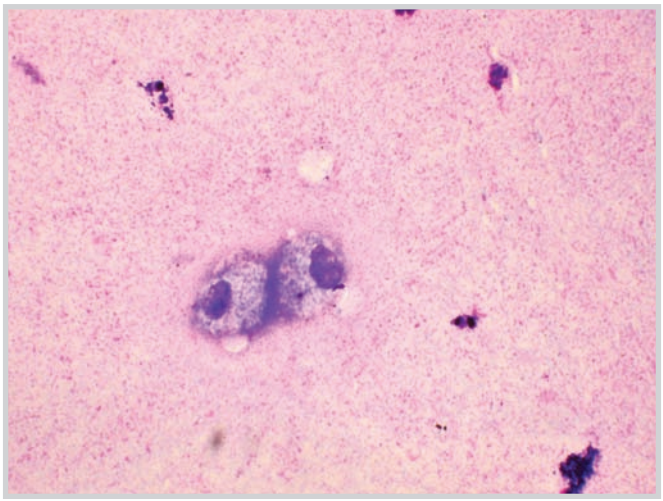


Fig. 10.28: Occasional round to oval cells in a background of thick mucinous material in pseudomyxoma pertonei (May-Grünwald-Giemsa XHP)

BOX 10.15 Pseudomyxoma peritonei

- Mucinous ascities and mucinous implants in the peritoneal surfaces
- Sources: Mucinous adenocarcinoma of ovary, mucocele of the appendix
- Cytology:
  - Thick and gelatinous mucoid materials
  - Round to columnar shaped cells
  - Bland nuclei
  - Clear cytoplasm
- Histology
  - Disseminated peritoneal adenomucinosis:
    - Superficial peritoneal implants
    - Focally proliferative mucinous epithelium
    - Little cytological atypia or mitotic activity
  - Peritoneal mucinous carcinomatosis
    - Invasive peritoneal lesions
    - Architectural and cytological features of carcinoma

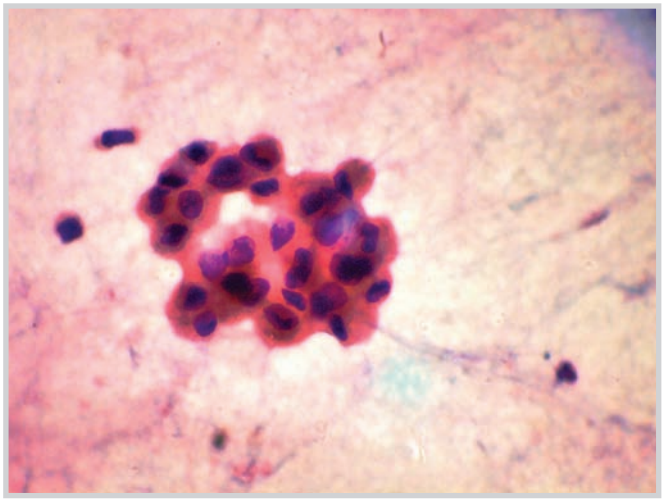


Fig. 10.29: Small loose cluster of cells in pseudomyxoma pertonei (Papanicolaou's stain X HP)

to 20% of malignant effusion is caused by—NHL and Hodgkin's lymphoma (HL).[8] Serous effusion caused by lymphoma has distinct sex and age variation. Male has higher frequencies of involvement by lymphomas than female.[9] In case of children, more than 50% of malignant effusion is caused by lymphoma and leukemia.[10,11] Altogether, involvement by the NHL in serous effusion is much more common than HL. T-cell NHLs are more frequently associated with effusion than B-NHL.[8]

Pleural effusion by lymphomas is mainly caused by:

1. Lymphatic obstruction due to pressure effect of thoracic duct
2. Direct infiltration of lymphoma on pleural surface
3. Radiation effect
4. Pulmonary infraction.[12]

Direct pleural infiltration is the predominant cause of effusion in cases of NHL, whereas, lymphatic obstruction is the main cause of effusion in HL.

Cytomorphology

Non-Hodgkin's lymphoma: Almost all types of NHL have been reported in effusion fluid. Cytomorphological features mainly depend on the primary type of NHL. The cells are always arranged discretely and they may frequently exhibit necrosis. Lymphoblastic lymphoma in effusion shows medium-sized cells with nuclear convolution and fine nuclear chromatin (**Fig. 10.30**). Burkitt's lymphoma shows cells with non-cleaved nuclei and prominent multiple nucleoli. The cytoplasm of

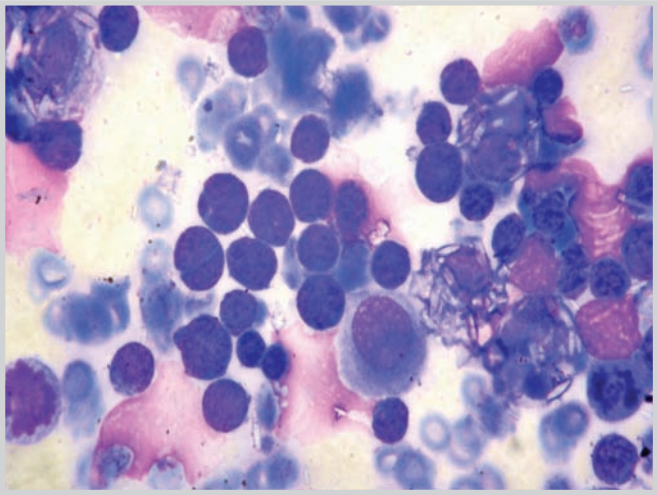


Fig. 10.30: Medium sized cells with scanty cytoplasm and reticular nuclear chromatin in a case of lymphoblastic lymphoma (May-Grünwald-Giemsa XHP)

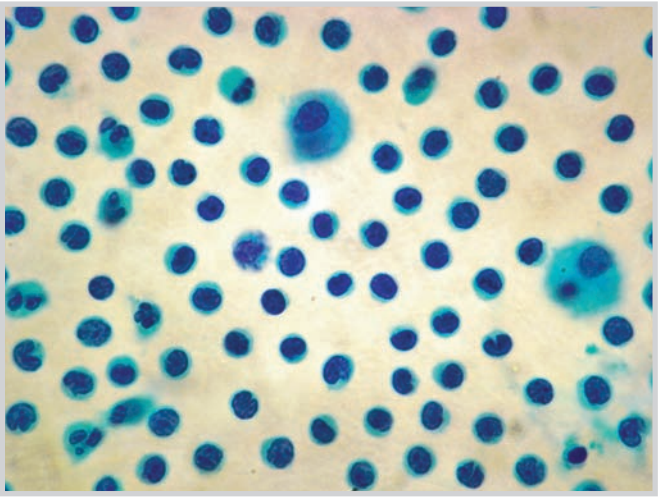


Fig. 10.31: Mixed population of small and large cells with nuclear cleaving in follicular center cell lymphomas (Papanicolaou's stain X HP)

the cell is deeply basophilic and vacuolated. The follicular center cell lymphomas show a mixed population of small and large cells with nuclear cleaving (**Fig. 10.31**). Anaplastic large cell lymphoma shows numerous medium to large lymphoid cells with frequent nuclear cleaving and large mononuclear and multinuclear cells**.** Doughnut cells with wreath-like arrangement are also noted. In case of small lymphocytic lymphoma or mantle cell lymphoma, the effusion cytology smear shows small cells with irregular clumped chromatin. Without any known history of lymphoma, it may be difficult to diagnose such cases as the cells may often be mistaken with reactive lymphoid cells**.** Monoclonal origin of the lymphoma cells can be demonstrated by flow cytometry.

Hodgkin's lymphoma: HL usually shows a polymorphic population of cells consisting of lymphocytes, plasma cells, and eosinophils. The typical Reed-Sternberg cell may be difficult to find out. A careful search of several smears may be needed to find out classical Reed-Sternberg cells. However, large mononuclear type of Hodgkin's cell and multinuclear cells may be noted in smear. In most of the cases of HL, the diagnosis is already known and therefore it may not be difficult to diagnose such cases.

Leukemia: In case of leukemic infiltration, it is very important to distinguish leukemic infiltration from admixture with peripheral blood in effusion fluid (**Fig. 10.32**). A differential count of peripheral blood and in effusion cytology smear may be helpful in this instance. The detailed cytological features of the leukemic cells are best demonstrable in the MGG stained smear.

Primary effusion lymphoma (PEL): Primary effusion lymphoma (PEL) or body cavity lymphoma is a distinct clinico-pathological entity which presents as serous effusion without any solid tumor mass (**Box 10.16**).[13,14] The PEL is a rare subtype of diffuse large B-cell lymphoma and is associated with human herpes virus 8 (HHV-8). It is predominantly found in advanced AIDS and cases with Kaposi sarcoma. The exact histogenesis and pathogenesis of this entity is still unclear. It has been suggested that vascular endothelial growth factor (VEGF) or vascular permeability factor (VPF) liberated by PEL cells may cause vascular leakage.[15]

Cytomorphology: The smears show large lymphoid cells with abundant basophilic cytoplasm and round nuclei with prominent nucleoli having a plasmablastic or immunoblastic differentiation. The cells may also have moderately pleomorphic, large, irregular multilobated nuclei indicating anaplastic differentiation (**Fig. 10.33**). Smears may show frequent mitosis and apoptosis. Rare cases of PEL with Burkitt's like lymphoma have also been described.[16] It is suggested that PEL is a unique subgroup of B-NHL. PEL cells usually express CD45 and positive for the following CD markers: CD19, CD20, CD23, CD38.[17,18] With the available history of immunodeficiency along with cytomorphology of the lymphoid cells, a possibility of PEL in effusion fluid should be suspected. Detailed immunocytochemistry and demonstration of HHV-8 are necessary for the final diagnosis.[19]

Differential Diagnosis

- *Anaplastic large cell lymphoma:* Large lymphoid cells with marked nuclear atypia may simulate ALCL. However, typical history of immunodeficiency and HHV-8 positivity may help in diagnosis of PEL.
- *Diffuse large B cell lymphoma:* The cells of Diffuse large B cell lymphoma (DLBCL) may simulate PEL. However, the presence of lymph nodal mass or any solid tumor mass and negative HHV-8 may exclude DLBCL.
- *Carcinoma:* The presence of large pleomorphic cells may simulate carcinoma in effusion. However, discrete cell population along with immunocytochemistry (CD45 positive) helps in exclusion of carcinoma.

Primary Serosal Tumor

Primary serosal tumors are uncommon. Mesothelioma and PEL are two malignant neoplasms that may arise from the serosal surface.

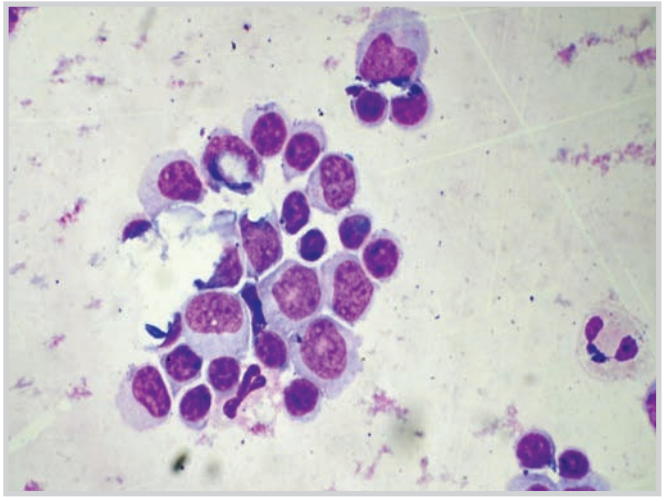


Fig. 10.32: Leukemic blasts in a case of leukemic infiltration (May-Grünwald-Giemsa X HP).

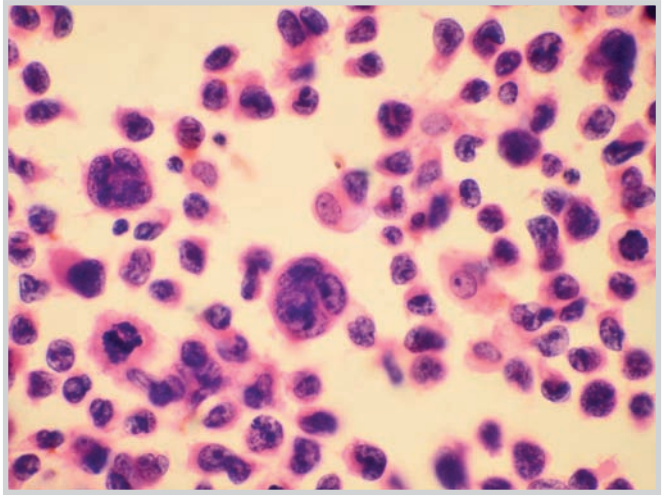


Fig. 10.33: Moderately pleomorphic, large, irregular multilobated nuclei in primary effusion lymphoma (Papanicolaou's stain X HP).

BOX 10.16 Primary effusion lymphoma

- Serous effusion without any solid tumor mass
- Noted in advanced AIDS and cases with Kaposi sarcoma
- Possibly a rare subtype of diffuse large B-cell lymphoma
- Associated with human herpes virus 8 (HHV-8)

Cytomorphology

Plasmablastic or immunoblastic differentiation

- Large lymphoid cells with abundant basophilic cytoplasm
- Round nuclei with prominent nucleoli

Anaplastic differentiation

- Moderately pleomorphic nuclei
- Large, irregular multilobated nuclei

Immunocytochemistry

Positive for: CD45, CD19, CD20, CD23, CD38

HHV-8 positive

Differential diagnosis:

- Anaplastic large cell lymphoma
- Diffuse large B cell lymphoma (DLBCL)
- Carcinoma

Mesothelioma

Mesothelioma can be diffuse and localized variety. Diffuse mesotheliomas are always malignant in nature. Localized mesothelioma may be either benign or malignant in nature. Only malignant mesothelioma causes effusion. It is to be noted that the epithelial variants of diffuse malignant mesothelioma have characteristic diagnostic features in effusion fluid.

Diffuse Malignant Mesothelioma

Diffuse malignant mesothelioma is often associated with asbestos exposure. It develops in the pleura, peritoneum, pericardium and tunica vaginalis. Pleural surface is the most common site of development of malignant mesothelioma (MM). It usually occurs in sixth or seventh decade of life. Initially, diffuse MM shows multiple tiny seed-like nodules that may not be detected in X-ray. Later on, the small nodules coalesce and large plaque-like nodule appears on the pleural or peritoneal surface. This is mostly seen in the basal or posterior surface of the lung. Later on, this may encase the whole lung. On histological examination, MM can be classified as epithelial, sarcomatoid or biphasic types.

The patient usually presents with chest pain, shortness of breath and unilateral pleural effusion.

Cytology (Box 10.17)

Cells in MM are arranged in three-dimensional spherical clusters with knob-like projections and papillae**.** Many discrete cells are also noted. Cells often show "cell in cell pattern" or cell cannibalism. For the cytological diagnosis of MM, primary duties of the cytologists are (1) to identify the cells as mesothelial in nature and (2) to decide the neoplastic nature of the mesothelial cells. The individual cells are round to oval with a window-like gap in between two cells (**Fig. 10.34**). Surface projections may also be seen. Cells usually show abundant dense cytoplasm with peripheral clear halo**.** Cells are enlarged with deceptively low nucleocytoplasmic ratio. Nuclei are usually central in position with frequent binucleation. Occasionally multinucleated cells are seen. Nuclei show mild pleomorphism with large prominent macronucleoli.

Differential Diagnosis

- *Metastatic adenocarcinoma:* MM may often simulate as metastatic adenocarcinoma and vice versa. In both the lesions, overlapping cytological criteria such as three-dimensional tight clusters, cytoplasmic vacuolations, moderately pleomorphic nuclei and prominent nucleoli are seen. However, there are a few subtle cytological differences in these two conditions. In MM, there is a striking absence of two cell populations and individual cells resemble like mesothelial cells with a window-like pattern, dense cytoplasm, and peripheral haloes. **Table 10.6** shows detailed distinguishing features between MM and metastatic adenocarcinoma.

BOX 10.17 Malignant mesothelioma

- Pattern:
  - Spherical clusters with knobby edges and papillae-like clusters
  - Dissociated cells
  - Cell in cell (cell cannibalism)
- Mesothelial cell morphology
- Window-like gaps in between the cells
- Enlarged cells
- Low nucleocytoplasmic ratio
- Frequent binucleation and multinucleation
- Centrally placed nucleus with large prominent nucleoli (macronucleoli)
- Condensed cytoplasm with ring-like peripheral clear zone

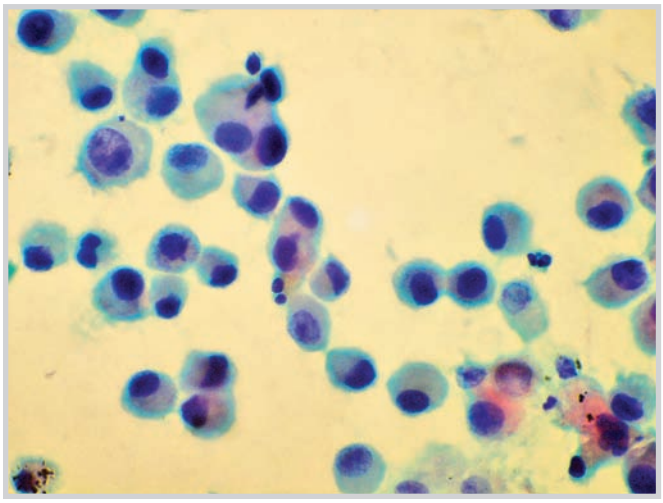


Fig. 10.34: Abundant malignant cells resembling mesothelial cells in a case of malignant mesothelioma (Papanicolaou's stain X HP)

TABLE 10.6: Distinguishing features between malignant mesothelioma and metastatic adenocarcinoma

Distinguishing points	*Malignant mesothelioma*	*Metastatic adenocarcinoma*
Two cell population	Absent	Present
Cell clusters	Spherical clusters with knobby edges	Hollow clusters
Cell in cell (cell cannibalism)	Frequent	Less common
Window like gaps	Present	Absent
Cytoplasmic peripheral halo	Present	Absent
Nucleo-cytoplasmic ratio	Low	High
Nuclear pleomorphism	Mild	Moderate
Nuclear position	Central	Central to eccentric
Multinucleated giant cells	More frequent	Less frequent
Cytochemistry Mucin stain (mucicarmine) Alcian blue	 Negative Positive and diastase sensitive	 Positive Positive and diastase resistant
Electron microscopy Intermediate filaments Microvilli	 Abundant Long	 Scanty Short
Immunocytochemistry Calretinin WT1 CEA Ber-EP4 Leu-M1 B72.3 Vimentin	 Positive Positive Negative Negative Negative Negative Positive	 Negative Negative Positive Positive Positive Positive Negative

Abbreviations: WT1—Wilms' tumor 1; CEA—Carcinoembryonic antigen

- *Reactive mesothelial cells:* Distinction between reactive mesothelial cells and MM is a great diagnostic challenge to the cytopathologist. The cells of MM look like mesothelial cells. Reactive mesothelial cells often show mild nuclear enlargement and pleomorphism. Moreover, the cells of MM may not show much nuclear enlargement. In this condition, clinical history of asbestos exposure, radiological evidences of nodular mass on the serosal surface along with other cytomorphological features such as a spherical cell cluster with a knobby appearance may be helpful. The cells of MM often show cytogenetic abnormalities such as deletion of 1p, 3p, 6q, 9p and 22q.[20]

ANCILLARY TECHNIQUES

The routine cytomorphological examinations are very helpful in the diagnosis of malignancy in most of the cases. Different studies have shown that the conventional cytology has near about 57% sensitivity and 89% specificity.[21] The positive and negative predictive values for detection of malignancies in effusion cytology are 89.3% and 69.4%, respectively.[21] However, there may be a grey zone where the cytologists face challenges in confirmation of diagnosis and subtyping of malignancies. In this condition, ancillary investigative techniques are helpful.

Immunocytochemistry

The diagnosis of malignancy in effusion fluid is primarily based on cytomorphology. However, the demonstration of various tumor markers in cells of effusion that are usually absent in non-neoplastic cells may be helpful in further confirmation.

In case of evaluation of effusion fluid, the cytologist has to face three primary challenges:

- Are the cells mesothelial or non–mesothelial in origin?
- Are the mesothelial cells malignant or benign?
- What is the type of non-mesothelial cells?

Mesothelial versus non-mesothelial: There are several markers which help in distinguishing mesothelial versus non-mesothelial cells. **Table 10.7** shows the different immunocytochemical markers or antibody to differentiate mesothelial cells from epithelial cells.

Carcinoembryonic antigen (CEA) positivity helps in elimination of mesothelial cell totally. In addition, Ber-EP4, Leu-M1 and B72.3 positivity also indicates epithelial origin (epithelial malignancy) of the cells. Carcinoma cells show diffuse keratin pattern whereas the mesothelial cells show dense concentric keratin positivity. Calretinin positivity indicates mesothelial origin of the cells. Mesothelial cells also show WT1, OV-CAR3, thrombomodulin, HBME-1 and N-cadherin positivity.[21]

TABLE 10.7: Markers of mesothelial cells and epithelial cells

Antibody	*Mesothelial cells*	*Epithelial cells*
CEA	Negative	Positive
BER-EP4	Negative	Positive
Leu-M1	Negative	Positive
B72.3	Negative	Positive
Calretinin	Positive	Negative
WT1	Positive	Negative
Low and high molecular weight cytokeratin	Both positive	Positive for low molecular weight keratin

Abbreviations: WT1—Wilms' tumor 1; CEA—Carcinoembryonic antigen

Malignant Versus Benign Reactive Mesothelial Cells

The separation of reactive mesothelial cells from malignant mesothelial proliferation is a major problem in the effusion cytology. Truly speaking, there is no immunohistochemical stain that firmly separates benign from malignant mesothelial proliferations. It has been suggested that epithelial membrane antigen (EMA) positivity is a sign of malignancy in epithelial mesothelial proliferations and strong EMA positivity indicates the presence of MM.[22] It has also been highlighted that strong p53 staining indicates malignant mesothelial cells.[23] However, p53 is not always positive in malignancies and unfortunately weak positivity may also be found in benign mesothelial cells.[24,25]

Hasteh F et al[25] applied a panel of immunocytochemistry consisting of EMA, Desmin, glucose-transport protein 1 (GLUT-1), Ki67, and p53 to distinguish reactive mesothelial cells and MM (**Box 10.18**). They noted that the combination of positive EMA and negative desmin strongly favored MM; conversely, a combination of negative EMA and positive desmin favored a reactive process. They also concluded that strong membranous positivity for GLUT-1 and/or strong nuclear staining for p53 favored a diagnosis of mesothelioma. No significance was noted in Ki67 proliferative index.

Type of Non-mesothelial Cells (Source and Type of Primary Tumor)

Immunocytochemistry greatly helps in categorization of the non-mesothelial cells and therefore the source of origin of the primary tumor. For the determination of primary source and type of tumor in effusion, following data are necessary (**Box 10.19**).

BOX 10.18 **Immunostain of malignant mesothelial cells**

- Strong epithelial membrane antigen positivity and negative desmin
- Strong membranous positivity for glucose-transport protein 1
- Strong nuclear staining for p53

BOX 10.19 **Important points to note before the use of immunocytochemistry for primary source or type of tumor**

- Any relevant history of primary
- Sex and age
- Location of the effusion
- Cytomorphology of the cells

Clinical History of the Patient

Age

Older age group patients usually show carcinomas and lymphomas. Occasionally, sarcomas and other rare tumors such as sarcomas may be seen.

Sex

Breast and ovarian carcinomas are more frequent in female patients whereas lung carcinomas are more frequent in male.

Location

As mentioned previously, location of effusion is very important. Certain tumors are more frequent in pleural than peritoneal effusion.

History of Previous Diagnosis of Malignancy

This is the most important clue to primary tumor's type.

Cytomorphology

As mentioned before, cytomorphological features are very helpful to decide the possible type of tumors.

Therefore, depending on the history one can apply, the panel of immunocytochemistry to identify the source or type of primary tumor. **Table 10.8** highlights the relevant immunocytochemistry for detection of primary source of the tumor.

TABLE 10.8: Immunocytochemistry for primary neoplasm detection

Primary	*Antibody panel*
Gastrointestinal tract	CEA, EMA
Ovary	CA-125, CEA
Prostate	PSA
Breast	Oestrogen and progesterone receptor
Melanoma	HMB-45
Thyroid	TTF, Thyroglobulin
Neuroendocrine	Synaptophysin, NSE, Chromogranin
Liver	Alpha-fetoprotein, Alpha 1 antyrypsisn
Lung	MOC-31, Ber-EP4, B72.3, CEA, TTF-1

Abbreviations: CEA—Carcinoembryonic antigen; EMA—epithelial membrane antigen; PSA—Periodic acid Schiff's; TTF—Thyroid tanscription factor

Flow Cytometry

DNA flow cytometry: Flow cytometric analysis of DNA content to detect aneuploid malignant cells in effusion sample has been done by several investigators. This technique has wide variation in sensitivity and specificity.[26-30] We have seen 59% sensitivity and 99% specificity of DNA FCM in effusion samples,[26] whereas others have shown 55%–100% sensitivity and 86%–100% specificity of DNA flow cytometry.[27-30] This wide variation of sensitivity and specificity has reduced the credibility of DNA flow cytometry significantly.

Ploidy analysis performed on a gated population of pan-cytokeratin positive cells yielded much better results. This helps in easier identification of DNA aneuploidy when the fluid is largely admixed with lymphocytes.[31] Therefore, we can say that single parameter DNA flow cytometry has limited utility in cytologically equivocal cases. Multiparameter flow cytometry by labeling of cells and DNA flow cytometry in a specific subset of cells may be more useful.

Flow Cytometric Immunophenotyping

Flow cytometry immunophenotyping (FCI) is usually applied as an ancillary technique for the diagnosis of hematological malignancies (**Box 10.20**). In comparison to immunocytochemistry, FCI is rapid, reproducible, sensitive, and reliable. Multicolor FCI can be used by a panel of antibody consisting of Ber-EP4, EMA, CD15 (epithelial markers), CD45 (leukocyte), and N-cadherin (mesothelial cell marker). Various authors have suggested that FCI is helpful in successful detection of epithelial cells in effusion cytology.[32,33]

Electron Microscopy

Transmission electron microscopy is helpful in differentiating MM from metastatic adenocarcinoma. Ultrastructurally, the mesothelioma cells shows abundant long, slender and branching microvilli devoid of glycocalyceal bodies, evenly distributed around the entire cell surface, whereas in adenocarcinoma these are short and stubby.[34,35] In addition, mesothelial cell shows abundant perinuclear tonofilaments, abundant glycogen, and apical tight junctions with well developed desmosomes.[35] Unfortunately, there may be overlapping features with poorly differentiated malignancies and a specific diagnosis may be difficult.

BOX 10.20 Flow cytometric immunophenotyping

- Rapid
- Reproducible
- Sensitive
- Reliable
- Markers:
  - Epithelial: Ber-EP4, EMA, CD15
  - Mesothelial: N-cadherin
  - Lymphoid: CD45
- Monoclonality:
  - B cell: Light chain restriction (Kappa or Lambda)
  - T cell: Aberrant expression of T cell markers

Abbreviations: EMA—epithelial membrane antigen

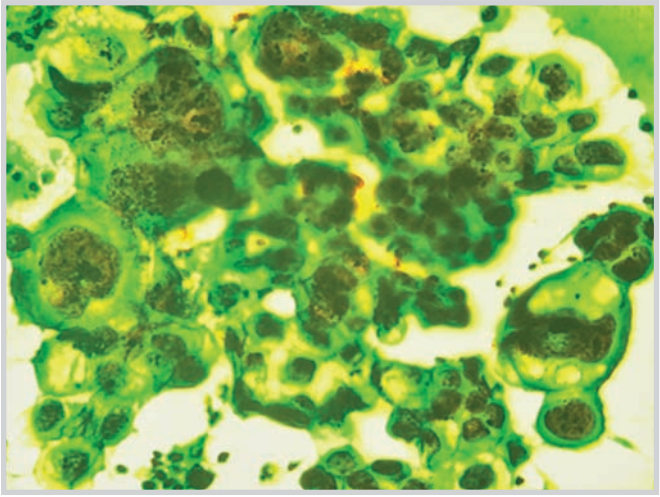


Fig. 10.35: Multiple large argyrophilic nucleolar organizer region (AgNOR) dots in malignant effusion (AgNOR stain X HP)

Argyrophilic Nucleolar Organizer Region

The argyrophilic nucleolar organizer region (AgNOR) technique detects specialized nucleolar protein by applying the silver impregnation method. The number and size of NORs indicate cellular metabolic and indirectly proliferation activity. Rapidly proliferating malignant cells show an increased number of large irregular NORs in comparison to less number of smaller NOR dots in reactive mesothelial cells[36–38] (**Fig. 10.35**).

Fluorescence In Situ Hybridization

Numerical chromosomal abnormalities are frequently present in the chromosomes of malignant cells and this can be demonstrated by fluorescence in situ hybridization (FISH) technique. FISH helps in microscopic identification, localization of aberrations in Interphase as well as metaphors of the cell cycle. Various centromeric probes have been used to demonstrate numerical chromosomal abnormalities for the detection of malignant cells. Field et al., by using centromeric probes representing chromosomes 7, 8, 11, 12, 17 and 18, detected significantly more malignancy in effusions than conventional cytology alone.[38,39] FISH can also detect hyperdiploid malignant cells and is particularly helpful when the malignant cells cannot be distinguished from reactive atypical cells.

Telomerase Activity

Telomeres are specialized repetitive DNA sequence at the ends of the chromosomes in eukaryotic cells. They are shortened with each cell division and finally critically shorten so that the cell enters into death process. The enzyme, telomerase, a ribonuclear protein, prevents telomeric loss and this enzyme is typically seen in neoplastic condition. Telomerase plays significant role in the development of various malignancies. A polymerase chain reaction (PCR) based method is applied to detect telomerase activity i.e., telomeric repeat amplification protocol (TRAP).[40] Various studies have shown increased telomerase activity in 52%–91% of pleural effusions, diagnosed as malignant in routine cytology.[41-43] It can be suggested that telomerase activity demonstrated by TRAP can be a valuable complementary technique in the assessment of malignancy in pleural effusions.

Image Morphometry

Computerized image analysis can estimate various morphometric parameters of the cell. The various cellular morphometric parameters and DNA content of the cells that can be measured by image analysis. It has been claimed that DNA image cytometry has high sensitivity and specificity.[44,45] However, the additional diagnostic value of this test is questionable.

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CHAPTER 11

Urine Cytology

Chapter Contents

INTRODUCTION

George Papanicolaou and Marshal[1] first time introduced urinary cytology for detection of malignancy. Later on, it was considered as a well-established technique for the detection of malignancy in the urinary tract. Despite of its low sensitivity, urinary cytological examination is still considered as a useful adjunct to the cystoscopy. It is an inexpensive, rapid and highly reliable technique for screening of lower urinary tract cancer. The major advantage of urine cytology is to detect the malignant lesions that are either not visible by cystoscopy or difficult to see on cystoscopy. The major disadvantages of urinary cytology are its low detection rate and failure to ascertain the exact anatomical localization of the lesion.

The major indications of urinary cytology are:

- To investigate the cause of hematuria
- Screening of recurrence of bladder cancer
- To screen asymptomatic high-risk cases for bladder cancer.

The most common presentation of bladder carcinoma is hematuria. A simple examination of urine sample can readily detect malignant cells. These cases should be further confirmed by cystoscopy and cystoscopic-guided biopsy.

Urine examination is particularly helpful in follow-up of patients with bladder cancer. High-grade urothelial carcinoma (UC) is prone to recur. Cystoscopy may be helpful in the detection of the recurrent cases. However, urine examination is particularly helpful in case of tumors that are not visualized by cystoscopy. It is a simple and inexpensive test for bladder cancer in asymptomatic people. Urine examination can be done for the high-risk asymptomatic people such as persons who are exposed to aniline dyes or cyclophosphamide therapy (**Box 11.1**).

ANATOMY

The lower urinary tract acts as a passage to transmit and store urine. It consists of renal pelvis, ureters, urinary bladder and urethra (**Fig. 11.1**).

Renal Pelvis

The renal pelvis is a funnel-like dilated portion of the proximal end of the ureters within the kidney. It receives the final product of urine from the renal calyces.

Ureter

The ureter is a muscular, tube-like, long, slender structure which transmits urine from the kidney to the bladder. Each

BOX 11.1 Urine cytology examination

Indications
- Investigation for hematuria cases
- Screening test for follow-up of bladder cancer
- Screening test for asymptomatic high-risk cases for bladder cancer

Advantages
- Simple
- Inexpensive
- Wide area of mucosal lesion is covered
- Especially helpful in lesions that are not visualized by cystoscopy
- High specificity

Disadvantages
- Low sensitivity particularly in low-grade urothelial tumors
- Exact anatomical location of tumor not possible

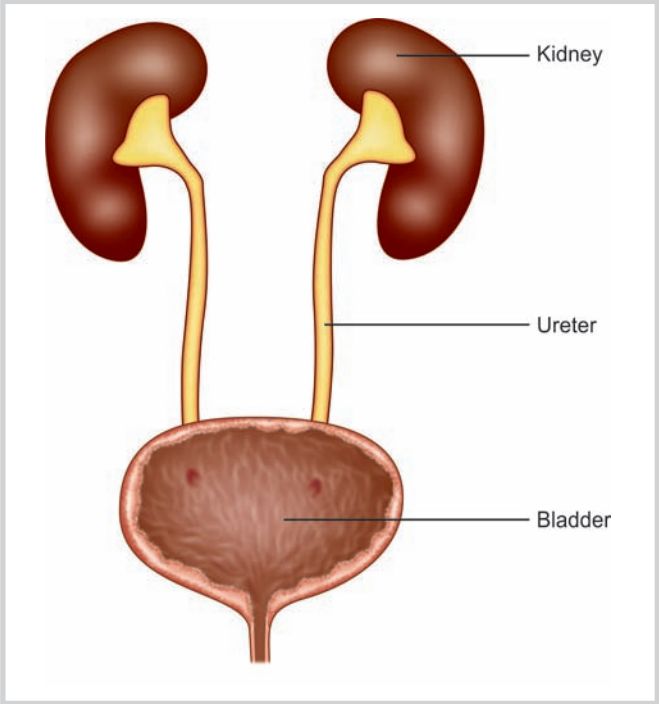


Fig. 11.1: Schematic diagram of the urinary tract

ureter is 25–30 cm long and 3–4 mm in diameter. The ureter is a retroperitoneal structure and passes through the abdominal cavity and then opens in the back of the urinary bladder.

Urinary Bladder

The urinary bladder is the muscular bag that receives urine from the two ureters and temporarily stores urine. It is superior to the prostate. In the male, the urinary bladder is situated in between the rectum posteriorly and pubic bone anteriorly. In females, the bladder is situated in between vagina posteriorly and pubic bone anteriorly. Urine exits from bladder via the urethra.

Urethra

Urethra is the tube that connects the urinary bladder to the outside of the body. In females, it is used for urination and in male urethra serves as a conduit of semen and also urine. Male urethra extends from the internal urethral orifice of the bladder to the external urethral orifice of the penis. It is about 17–20 cm long. The female urethra is a membranous tube 4–5 cm long and extends from the internal urethral orifice of the urinary bladder to the external genitalia in between the clitoris and vagina.

HISTOLOGY

Ureter

Ureter has three layers:
1. Inner mucosal layer—Mucosa: Mucosa of the ureter is lined by 3–5 layers of transitional epithelium and is rested on dense fibroconnective tissue, known as lamina propria.
2. Muscular layer: Muscle layer consists of inner longitudinal, middle circular and outer longitudinal layer.
3. Outer fibroconnective tissue coat.

Urinary Bladder

Urinary bladder has three layers: (1) inner mucosal layer, (2) submucosal layer and (3) muscular layer.

1. Inner mucosal layer: Mucosa of the urinary bladder is lined by 5–6 layers of transitional epithelial cells (**Fig. 11.2**). There are three different zones of cells: basal cell layer, intermediate cell layer and surface cell layer. The basal layer is composed of one cell layer of cuboidal epithelial cells. The intermediate cell layers are about five layers thick and the cells are more columnar with their nuclei orientated at right angles to the basement membrane. The superficial layer is only one layer thick. These cells are also known as umbrella cells or dome cells and have the unique characteristics that allow them to maintain impermeability to urine to mucosal layer. The cells are large and oval in shape having abundant eosinophilic cytoplasm and round centrally placed nuclei. The surface outline of the cell is fuzzy and indistinct. Many cells are binucleated and a few are multinucleated. The superficial transitional epithelial cells have certain unique ultrastructural features that help them to expand greatly and rapidly when the bladder is distended with urine and the epithelium is at full stretch. The plasma membrane of the umbrella cells have specialized, rigid, thickened regions, known as plaques (also called as asymmetrical unit membrane). These plaques are interspersed with narrow zones of normal interplaque regions. The plaques probably play an important role in maintaining urine-blood barrier and intervening interplaque parts take part in the expansion of the bladder wall during the stretch.

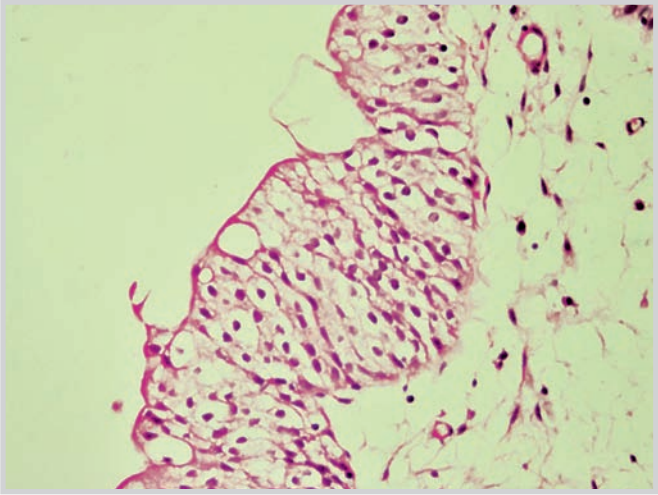


Fig. 11.2: Histology section shows transitional epithelial layer of bladder mucosa (Hematoxylin and Eosin X MP)

2. Submucosal layer: Mucosal membrane of the bladder rests on the submucosal layer. It consists of connective tissue and dense capillary plexus. This capillary plexus serves not only as vascular supply but it also acts as a urinary-blood barrier.
3. Muscular layer: The muscle layer consists of innermost longitudinal, middle circular and outermost longitudinal layers that are often difficult to distinguish.

Urethra

The female urethra is lined by transitional epithelium near the bladder and the rest of the part is lined by nonkeratinized stratified squamous epithelium with intervening pseudostratified columnar epithelium.

The prostatic part of the male urethra is lined by transitional epithelium. The cavernous urethra is lined by stratified columnar epithelium interspersed with portions of the pseudostratified columnar epithelium. The penile urethra is predominantly lined by stratified and pseudostratified columnar epithelium, except near the meatus that is lined by stratified squamous nonkeratinized epithelia.

NORMAL CYTOLOGY

The normal voided urine sample shows scanty cells. The predominant cell population is transitional cells. However, the other cells may also be noted (**Box 11.2**).

Transitional Cells

The cells are usually present in discrete or in small loose clusters.

Voided urine sample usually shows discrete superficial cells whereas, the catheterized specimen shows clusters or papillary groups of cells containing intermediate and parabasal cells.

BOX 11.2 **Normal cells in urine**

- Urothelial cells
  - Superficial umbrella cells
  - Intermediate cell
  - Basal cells
- Reactive transitional cells
- Columnar cells
- Squamous cells
- Others
  - Renal tubular cells
  - Seminal vesicle cells
  - Prostatic cells
  - Blood cells: polymorphs, lymphocytes, histiocytes, giant cells, etc.

BOX 11.3 **Superficial umbrella cells**

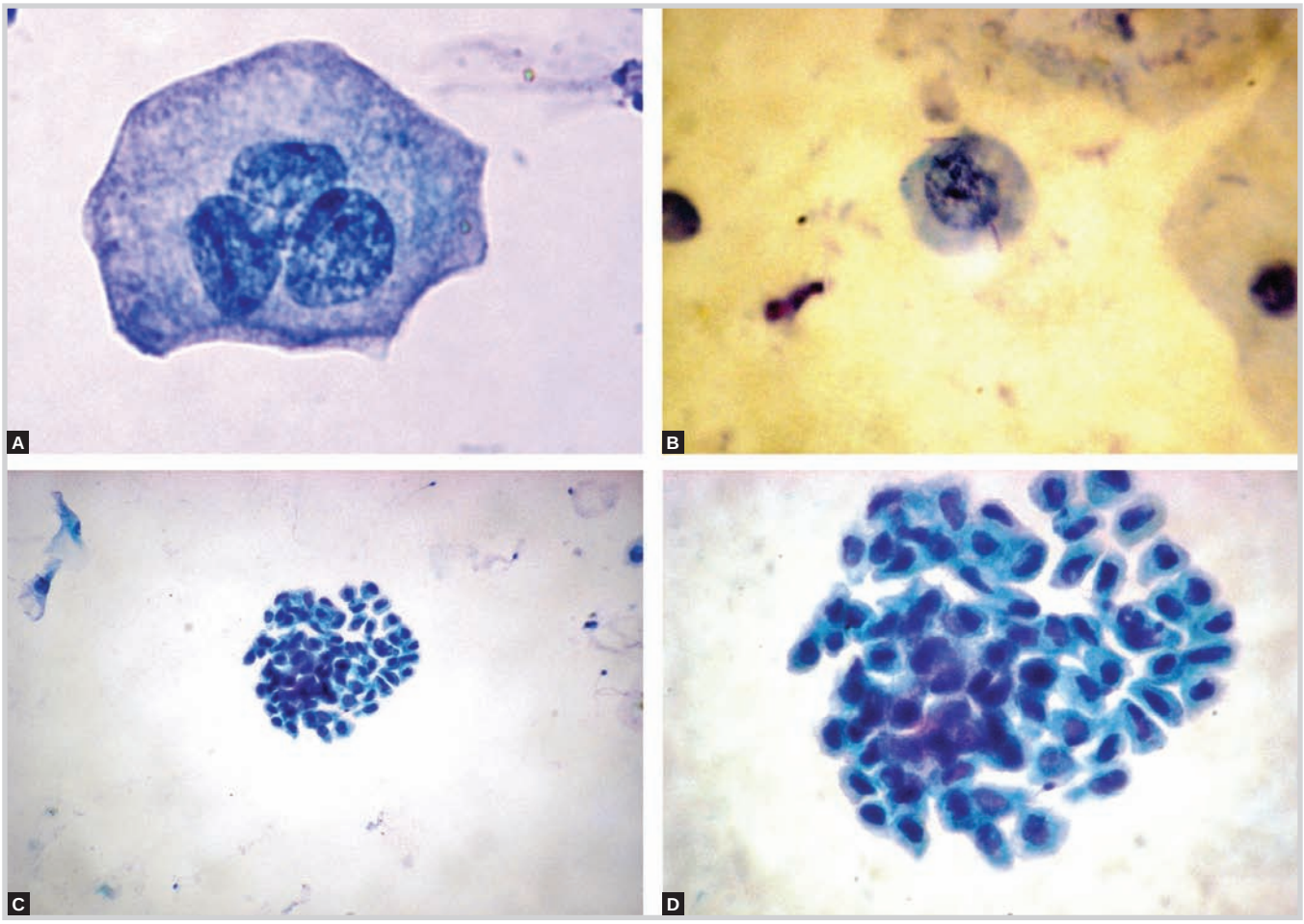
- Large cell
- Abundant cytoplasm
- Well-defined cytoplasmic margin
- Centrally placed nucleus
- Very low N/C ratio
- Finely granular nuclear chromatin
- Frequent binucleation and multinucleation

Superficial Cells (Umbrella Cells)

Superficial cells are the largest epithelial cells in our body. They are also known as umbrella cells as they protect the underneath cells from the toxic urine of bladder (**Box 11.3**). These cells are about 100 micron in diameter. The cells are round to oval in shape with abundant eosinophilic finely granular cytoplasm. The cytoplasmic margin of the cell is well-defined. The nucleus is round and central in position with very low nuclear-cytoplasmic (N/C) ratio (**Fig. 11.3A**). The chromatin is granular with inconspicuous nucleoli. The umbrella cells are often bi or multinucleated and may show even 10–20 nuclei. At times, the superficial cells may show mild nuclear hyperchromasia and pleomorphism. However, the cells maintain low N/C ratio and are usually easily recognizable from neoplastic cells. Degenerated urothelial cells often contain large round eosinophilic cytoplasmic inclusions that are known as Melamed-Wolinska bodies. These structures are seen more frequently in catheterized urine sample than voided urine sample.

Intermediate Cells

The basal/parabasal cells are small round to oval cells with well-defined cell margin and scanty cytoplasm that often shows diffuse fine vacuoles. The nuclei are relatively larger with evenly distributed fine granular chromatin (**Box 11.4**). The intermediate



Figs 11.3A to D: (A) Multinucleated umbrella cell on urine cytology smear (Papanicolaou's stain X HP); (B) Intermediate cell in urine cytology smear (Papanicolaou's stain X HP); (C) Reactive atypia of transitional cells in urine cytology smear (Papanicolaou's stain X MP); (D) Reactive atypia of transitional cells in urine cytology smear showing nuclear enlargement and pleomorphism (Papanicolaou's stain X HP)

cells are a bit larger compared to basal cells. The cells show basophilic cytoplasm with central round nuclei and prominent nucleoli (**Fig. 11.3B**). Bi and multinucleated cells are infrequent as compared to superficial cells.

Reactive Transitional Cells

Reactive transitional cells are commonly seen in bladder stone, viral or bacterial cystitis, catheterization or other instrumentation and radiation or drugs (**Box 11.5**). The superficial transitional cells show nuclear enlargement, mild nuclear pleomorphism, hyperchromasia and even coarse chromatin (**Figs 11.3C** and **D**). Nuclear membrane may be thickened focally. There may be prominent nucleoli. Low-grade transitional cell carcinoma (TCC) usually does not show prominent nucleoli. Murphy et al.[2] have described such cells and emphasized to differentiate the reactive transitional cells from high-grade TCC. The N/C ratio of the reactive transitional cells is usually not high compared to high-grade TCC. The markedly coarse chromatin and hyperchromasia in TCC may also be helpful cytological features to differentiate these two conditions.

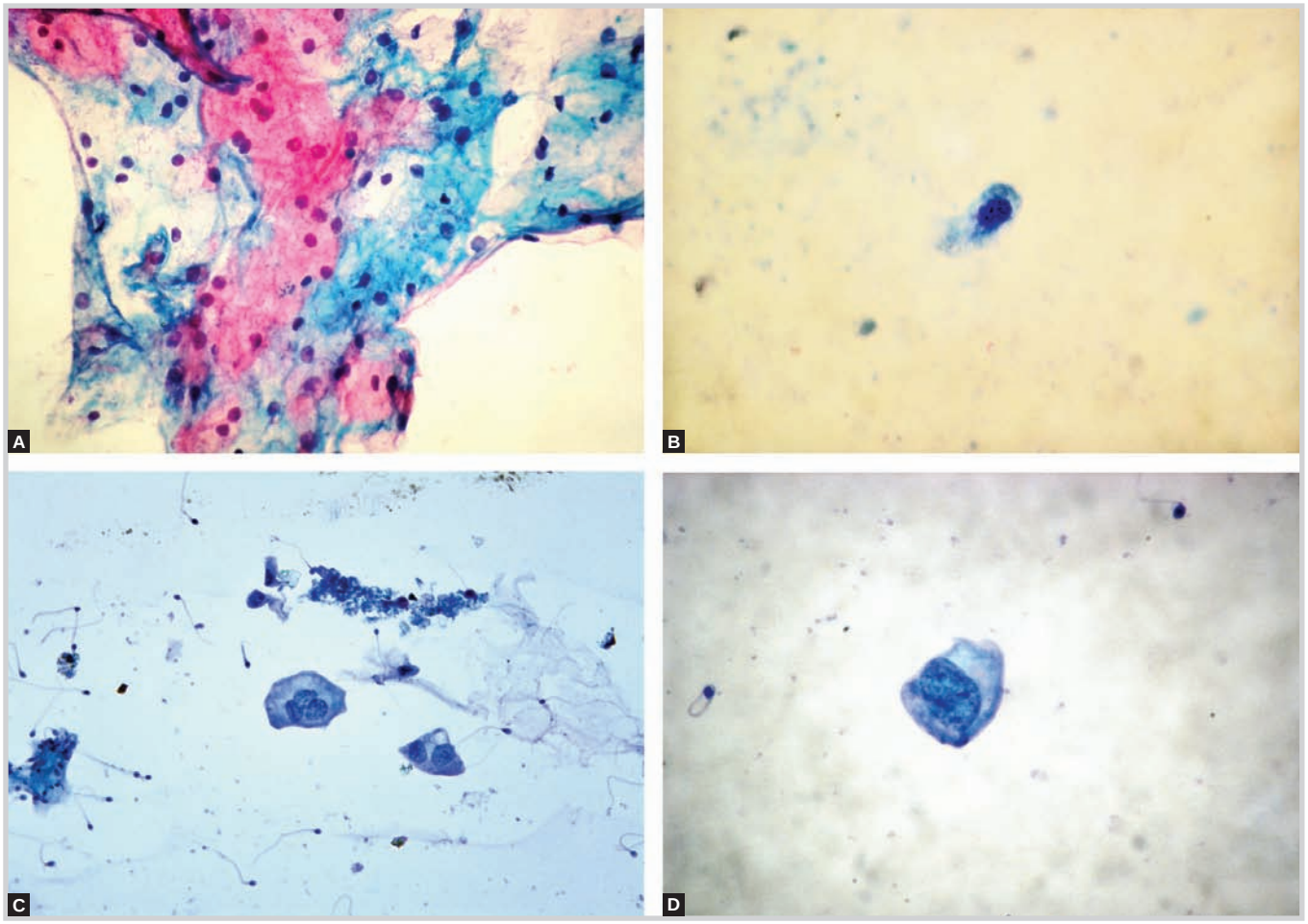
BOX 11.4 Intermediate and parabasal cells

- Smaller cells
- Round to oval
- Diffuse fine vacuoles in the cytoplasm
- Central to eccentric round nuclei
- One to two nucleoli
- Bland chromatin

BOX 11.5 Reactive transitional cells

- Round to oval cells
- Enlarged nucleus with increased N/C ratio
- Mild nuclear pleomorphism
- Mild hyperchromasia
- Prominent nucleoli
- Vacuolated cytoplasm

Commonly seen: Stone, cystitis, instrumentation, radiation, and drugs



Figs 11.4A to D: (A) Benign squamous epithelial cells in urine cytology smear (Papanicolaou's stain X MP); (B) Columnar cell in urine cytology smear (Papanicolaou's stain X HP); (C) Many spermatozoa in urine cytology smear (Papanicolaou's stain X HP); (D) Seminal vesicle cell in urine cytology smear (Papanicolaou's stain X HP)

BOX 11.6 **Squamous cells**

- Commonly seen in urine
- Both superficial and intermediate squamous cells
- Common sources are trigone of the bladder, meatus of penis and vaginal contamination
- Other sources: Stone, infection such as schistosomiasis, exogenous hormone treatment

Squamous Cells

Squamous cells are commonly seen in urine (**Box 11.6**). Both superficial and intermediate types of squamous cells may be noted. The squamous cells are commonly exfoliated from squamous metaplasia of trigone of the bladder **(Fig.11.4A)**. This is more frequent in females than males. In addition, the squamous cells may come from the terminal part of urethra as this is lined by squamous epithelium. The other sources of squamous cells are lithiasis, infections and inflammation.

Columnar Cells

The presence of columnar cells in voided urine is uncommon, however, these cells are frequently present in the catheterized urine sample (**Box 11.7**). They are also present due to response to chronic irritation of the bladder. The other sources of columnar cells are cystitis cystica, the lacunae of Morgagni and the glands of Littre of the urethra, seminal vesicles, prostatic epithelium, renal tubules and rarely from endometrium in endometriosis.

The cells are elongated, columnar in shape with moderate amount of cytoplasm and basally placed nuclei. The cytoplasm of the cells may have small vacuoles (**Fig. 11.4B**).

Pseudopapillary Clusters of Urothelial Cells

Clusters of urothelial cells forming papillae-like structures are frequently seen in catheterized urine samples, bladder washing, lithiasis and cystitis (**Box 11.8**). The pseudopapillary arrangement of the cells may often pose diagnostic difficulty

BOX 11.7 Columnar cells

- Commonly seen in
  - Instrumentation
  - Cystitis cystica
  - Renal tubules
  - Intestinal metaplasia
  - Periurethral glands
  - Endometriosis
- Morphology
  - Columnar in shape
  - Moderate amount of cytoplasm
  - Small vacuoles
  - Basally placed nuclei

BOX 11.8 Papillary clusters

Causes
- Instrumentation
- Inflammatory injury
- Bladder catheterization
- Stone
- Urothelial carcinoma

Distinguishing points in favor of benign lesions
- Margin of the papillae is smooth and regular
- Discrete cells have monomorphic and normochromatic nuclei

with TCC. However, the peudopapillary cluster in reactive condition is ball-shaped, cohesive with smooth margins. The cytomorphology of the discrete cells is also very important to differentiate the reactive condition from TCC.

Other Cells

There may be sparse polymorphs in the normal urine. Histiocytes, lymphocytes and plasma cells are present in inflammatory condition. Sperms are seen in urinary sample after a prostatic massage or retrograde ejaculation (**Fig. 11.4C**).

Seminal vesicle cells, epididymal cells, prostatic epithelial cells and cells from ileal loops may also be present at times (**Fig. 11.4D**).

SPECIMEN COLLECTION

There are mainly three types of specimen collection techniques of urine:

1. Voided urine specimen
2. Catheterized specimen
3. Bladder wash and brush cytology

Voided Urine Specimen

Voided urine collection is the specimen of choice for screening in a population with specific symptoms such as hematuria (**Box 11.9**). The urine sample should be collected 3–4 hours after last micturition. First morning voided urine should better be avoided as it shows considerable amount of degeneration because of prolonged stagnation.[2,3] Fresh randomly voided urine specimen should be collected in a clean container and should be sent to a laboratory for immediate processing to avoid degenerative changes. About 50 mL of urine is sufficient for cytological examination.

Voided urine is easy to collect; however, the urine sample shows sparse cellularity and considerable degenerative changes. In addition, in female patients there may be high chances of contamination from vaginal cells.

Catheterized Urine

A catheterized urine sample is used in cases of follow-up of a patient of known neoplastic lesion of the lower urinary tract (**Box 11.10**). Catheterized urine sample yields better cellularity and shows less degenerative changes. However, it may cause breaking up of large tissue fragments that may simulate malignancy. Another important disadvantage of this technique is introduction of urinary tract infection by the tip of the catheter.

Bladder Wash and Brush Cytology

Bladder wash and brush are indicated in cases of suspected urothelial neoplasm or follow-up of a case of known neoplastic lesion (**Box 11.11**). The bladder wash specimen is obtained by rinsing the bladder with 50 mL of sterile normal saline for 3–4 times. The collected fluid is processed. Bladder brushing is done by direct supervision under cystoscopy. The main advantages of this technique are: (1) good cellularity, and (2) better preservation of the cells. The major disadvantages are: (1) instrumental artifact, (2) invasive technique and (3) uncomfortable to the patient.

Other Sampling Techniques

Ureteric Sample

Urine may also be collected by introducing a catheter to the ureter. Urine can be collected separately from each ureter to localize the specific pathology in the ureter or kidney. Smear from each side can be compared for subtle cytological abnormalities.

Ileal Conduits

In case of total cystectomy, surgeon makes a duplicate bladder with the help of ileum to provide a conduit of urine. Urine samples are often taken to examine the recurrence of bladder cancer. The sample from ileal conduit usually contains lot of intestinal epithelial cells.

BOX 11.9 **Voided sample**

- Choice of sampling in screening of symptomatic patient

Collection
- Three to four hours after last urination with adequate hydration
- Fresh urine in clean container
- At least 50 mL urine
- No preservative

Advantages
- Easy technique
- No artifact due to instrumentation
- Noninvasive technique

Disadvantages
- Degenerative changes
- Contamination of vaginal cells in female

BOX 11.10 **Catheterized urine**

Indication
- Follow-up of a patient of known neoplastic lesion of the lower urinary tract

Advantages
- Good cellularity
- Better preservation than voided urine sample

Disadvantages
- Instrumental artifact: Large papillary clusters of cells
- Urinary tract infection
- Invasive

BOX 11.11 **Bladder wash**

Indications
- Suspected urothelial neoplasm
- Follow-up of a case of known neoplastic lesion

Advantages
- Good cellularity
- Better preservation of the cells

Disadvantages
- Instrumental artifact
- Invasive technique
- Uncomfortable to the patient

PROCESSING

The urine sample should be processed immediately after receiving the specimen. Urine is processed by any of these techniques depending on the amount and availability of the technique in the laboratory:

Simple Centrifugation

Approximately 50 mL urine is centrifuged for 10 minutes with 1,200 rounds per minute. Multiple smears are prepared from the sediment and alcohol-fixed smears are kept for staining.

Millipore Technique

Large amount (more than 200 mL) of clear urine is processed by this technique.

Cytocentrifugation

Small amount of clear urine is processed by cytocentrifugation.

Liquid-based Preparation

Liquid-based preparation may be used in urine specimen preparation.[4] This technique provides better morphology and cell preservation along with better cellularity. However, it may not be a cost-effective technique.

Cell Block

If a fleshy tissue is visible in urine specimen, then cell block could be done.

NON-NEOPLASTIC LESIONS IN THE URINARY TRACT

Lithiasis

The patient of urinary lithiasis may present with hematuria and pain. However, many patients may be totally asymptomatic **(Box 11.12)**.

BOX 11.12 **Urinary calculi**

Symptoms: Pain and hematuria or asymptomatic

cytology
- Papillary clusters
- Smooth border of papillae
- Cells with centrally placed nuclei
- Predominantly normal nuclear size and shape but mild nuclear enlargement may be seen
- Regular nuclear membrane
- Nuclear hyperchromasia
- Prominent nucleoli
- Squamous metaplasia
- Stone fragments
- Acute inflammatory cells

Differential diagnosis: Transitional cell carcinoma

Radiologically, there may be filling defect in the bladder. Urinary lithiasis is a common cause of false positive diagnosis as cytological features of urinary lithiasis may often be confused with malignancy.[5] The smears are hypercellular with large fragments of urinary epithelial cells. The cells are often sloughed as papillary clusters. The papillae have usually smooth borders unlike ragged margin of the papillae of TCC (**Box 11.13**). The individual cells may show mild nuclear atypia and may mislead the cytologists. The individual cells have centrally placed nuclei with mild nuclear enlargement and pleomorphism (**Fig. 11.5**). The nuclear chromatin is coarsely clumped and dark. Nucleoli are prominent. Metaplastic squamous cells may be seen in the smears. In addition, background shows acute inflammatory cells, blood and necrosis. This may simulate tumor diathesis. Fragments of stone may also be noted as calcified and laminated structures.

Bacterial Infection

Cystitis

Most cases of bacterial cystitis are due to Gram-negative organisms particularly *Escherichia coli*, *Proteus, Klebsiella,* and *Pseudomonas aeruginosa*. Gram-positive infections rarely cause cystitis. The patient usually presents with dysuria, increased frequency of micturition and suprapubic tenderness. The smears show abundant polymorphs, RBCs and necrotic debris. The urothelial cells may be obscured by the inflammatory cells. The transitional cells often show reactive atypia with mild nuclear enlargement, pleomorphism, hyperchromasia and prominent nucleoli (**Fig. 11.6**).

BOX 11.13 **Similarity and dissimilarity of lithiasis and transitional cell carcinoma**

Simulating features
- Both lithiasis and TCC show papillary clusters
- Nuclear atypia
- Nuclear hyperchromasia and coarse chromatin
- Necrosis, blood and polymorphs stimulate tumor diathesis

Distinguishing points

In lithiasis
- Papillae are usually smooth bordered
- Prominent nucleoli
- Stone fragments
- Nuclear atypia is not so severe
- Clinical history of stone such as pain or radiological features

Differential Diagnosis

Transitional Cell Carcinoma

Reactive atypia in urothelial cells should always be distinguished from that of TCC. The presence of abundant polymorphs, mild nuclear atypia and degenerative changes in nucleus usually indicate reactive changes. In the presence of chromatin smudging or poor preservation of nucleus, one should always be careful in the diagnosis of TCC.

Fungal Infections

Fungal infections are commonly seen in diabetic patients and other immunocompromised patients. *Candida* is the most common fungal infection and other fungal infections are *Aspergillus, Histoplasma* and *Cryptococcus*. Cytological features of fungal infections are similar to that of bacterial infections. The presence of candidal spores in the absence of inflammatory reaction usually indicates contamination in urine.

Viral Infections

Human Polyomavirus

Human polyomavirus is also known as BK virus. It is a DNA virus (**Box 11.14**). BK virus is the member of papova virus family. It commonly infects the lower urinary tract with apparently no clinical significance. They commonly affect the patients with renal transplant, steroid therapy, cancer and AIDS. However, BK virus infection may produce cytological changes in the urinary

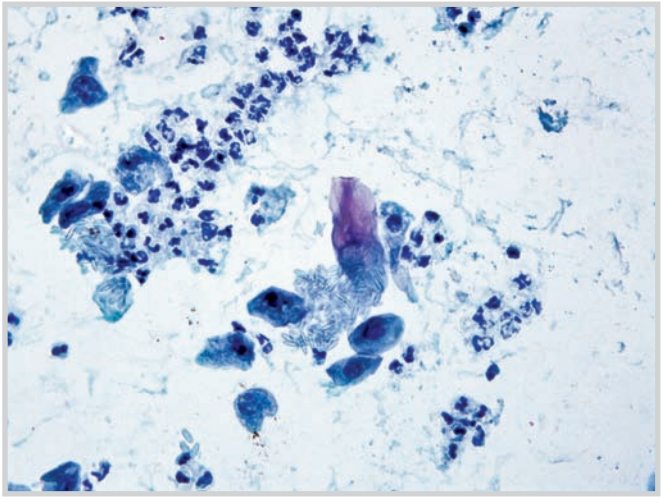


Fig. 11.5: Atypical cells in urinary lithiasis along with crystals (Papanicolaou's stain X HP)

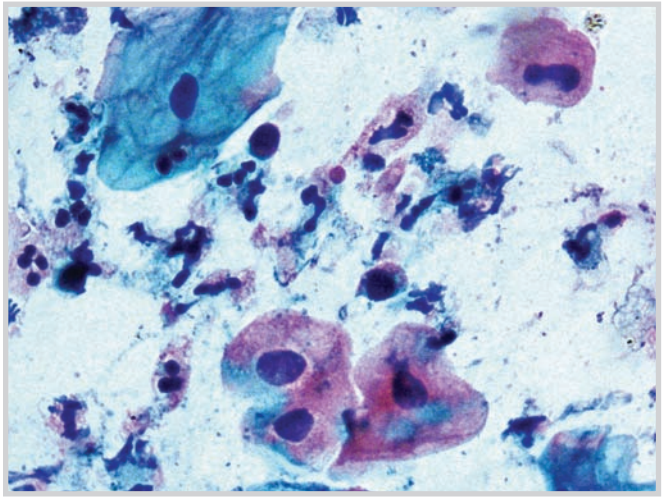


Fig. 11.6: Polymorphonuclear leukocytes with atypical urothelial cells in urine (Papanicolaou's stain X HP)

BOX 11.14 Human polyomavirus

- BK virus: DNA virus, papova family
- Commonly seen in renal transplant patients, cancer patients, steroid therapy
- Enlarged cells with eccentric nuclei giving a comet-like appearance (comet cell)
- Cells with nuclear enlargement, high N/C ratio, hyperchromatic nuclei, irregular nuclear membrane (decoy cells)
- Large basophilic intranuclear inclusion body
- Smudgy nucleus

Differential diagnosis: Transitional cell carcinoma

tract that may simulate TCC.[6] The cells are usually enlarged with eccentric nuclei giving a comet-like appearance (comet cell). Individual cells show nuclear enlargement, high N/C ratio, hyperchromatic nuclei and irregular nuclear membrane. The nucleus may have a large basophilic inclusion body that completely replaces the nuclei and makes it smudgy (**Fig. 11.7**). The inclusion may completely replace the nucleus and only a peripheral rim of the nucleus may remain intact. As the infected urothelial cells simulate high-grade TCC so they are also known as "decoy cells".

Human Papillomavirus

These are DNA viruses and commonly infect urothelial lining of bladder and squamous lining of cervix.[7] The patient commonly presents with condylomatous lesion of bladder. Cells with koilocytic changes are diagnostic. In voided urine of female patients, the possibility of contaminated cells from the lower genital tract should always be excluded.

Cytomegalovirus

It commonly infects the renal convoluted tubular cells. The infected cells are rarely seen in urine specimen. The cells are enlarged with large nuclei having a large intranuclear inclusion and perinuclear clearing.

Chemotherapeutic Effect

Chemotherapeutic drugs like cyclophosphamide, busulfan, thiotepa and mitomycin may show significant changes of the urothelial cells that simulate malignancy.

Some drugs like cyclophosphamide and busulfan are introduced systemically and are concentrated in the bladder. Other drugs like mitomycin C, thiotepa, adriamycin, etc. are used locally.

Cyclophosphamide

The active metabolite of cyclophosphamide is concentrated in the urine and produces hemorrhagic cystitis. There is necrosis and sloughing out of the bladder mucosal lining resulting in profuse hematuria. On cytological examination, the urothelial cells show nuclear enlargement with high N/C ratio (**Fig. 11.8**). Nuclei show mild pleomorphism, irregular nuclear outline, dark coarse chromatin and large prominent nucleoli. These atypical cells closely mimic high-grade TCC. The cells often show considerable nuclear degenerative changes.

Differential Diagnosis

- *High-grade transitional cell carcinoma:* Significant nuclear atypia of the cells may mislead to the diagnosis of TCC. Without proper clinical history, it may be impossible to diagnose cells with cyclophosphamide effect.
- *Viral Infection:* Enlarged atypical nucleus may often simulate polyomavirus infection.

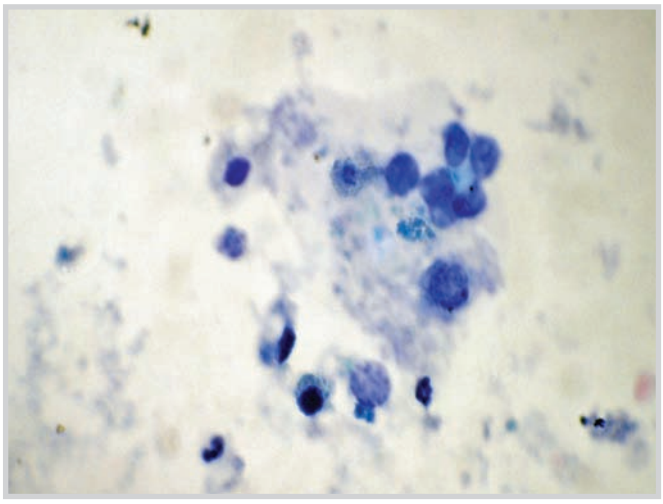


Fig. 11.7: Decoy cells in urine (Papanicolaou's stain X HP)

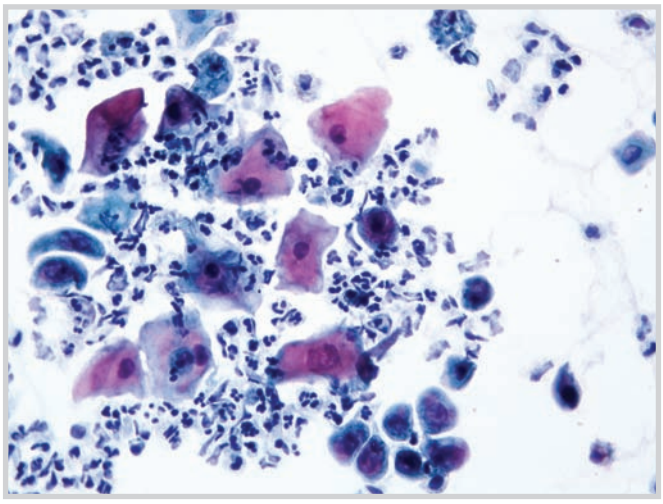


Fig. 11.8: Chemotherapy induced changes in urine. Nucleus is enlarged with smudgy outline (Papanicolaou's stain X HP)

Busulfan

Busulfan therapy usually affects lung and occasionally it may cause hemorrhagic cystitis. The cells show nuclear enlargement, pleomorphism and hyperchromasia. These cells simulate high-grade TCC.

Mitomycin C and Thiotepa

These drugs are locally used intravesically. They also cause sloughing and degeneration of both normal and neoplastic urothelial cells. Urothelial cells show nuclear enlargement and pleomorphism. However, the N/C ratio of the cells is not altered. Nuclei often show multiple small nucleoli. Cytoplasm shows polychromasia and vacuolations.

Bacillus Calmette-Guérin Vaccine

Immunotherapy with the attenuated *Mycobacterium bovis* strain, Bacillus Calmette-Guérin (BCG) is currently used as chemotherapeutic agents for bladder cancer particularly carcinoma in situ (**Box 11.15**). Cytological examination of urine of the patients initially shows neutrophilic reaction along with degenerated urothelial cells. Later on lymphocytes and histiocytes appear. The smear often shows a loose collection of histiocytes and ill-formed epithelioid cell granulomas (**Figs 11.9** and **11.10**).[8] Individual cells may show mild nuclear enlargement and hyperchromasia. The N/C ratio of these cells is usually maintained.

Radiation Effect

Pelvic irradiation is commonly used in cervical cancer, bladder cancer and other pelvic malignancies. Radiation causes mucosal edema, congestion and ulceration. The urothelial cells show degenerative changes (**Box 11.16**).

Cytology smear shows markedly enlarged cells known as macrocytes. Both cytoplasm and nucleus are enlarged and therefore there is no change of N/C ratio. Cytoplasm shows polychromasia or eosinophilia and contains multiple degenerative vacuoles. Nuclei also show vacuoles, mild hyperchromasia and multiple prominent nucleoli. Nuclear membrane is usually regular. Nuclear chromatin may be smudgy and featureless. Chronic radiation effect in urine may show multinucleated giant cells and chronic inflammatory cells (**Fig. 11.11**).

BOX 11.15 **Bacillus Calmette-Guérin therapy**

- Attenuated *Mycobacterium bovis* strain used for carcinoma in situ of bladder
- Initial: polymorphs, degenerated urothelial cells
- Later stage: Lymphocytes, histiocytes
- Epithelioid cell granulomas
- Multinucleated giant cells
- Atypical transitional cells: Nuclear enlargement, hyperchromasia
- No change in N/C ratio

Differential Diagnosis

Malignant Cells

Radiation changes may simulate malignant cells because of markedly enlarged cells and nuclear hyperchromasia. However, the cells in radiation effect do not show any alteration of N/C ratio. Both cytoplasm and nuclei of these cells show degenerative vacuoles. Nuclei show smudged featureless chromatin.

BOX 11.16 **Radiation changes**

- Marked cytomegaly (macrocytes)
- Nuclear enlargement
- No alteration of N/C ratio
- Cytoplasmic eosinophilia and vacuoles
- Nuclear vacuoles
- Prominent and multiple nucleoli (macronucleoli)
- Nuclear hyperchromasia
- Multinucleated cells

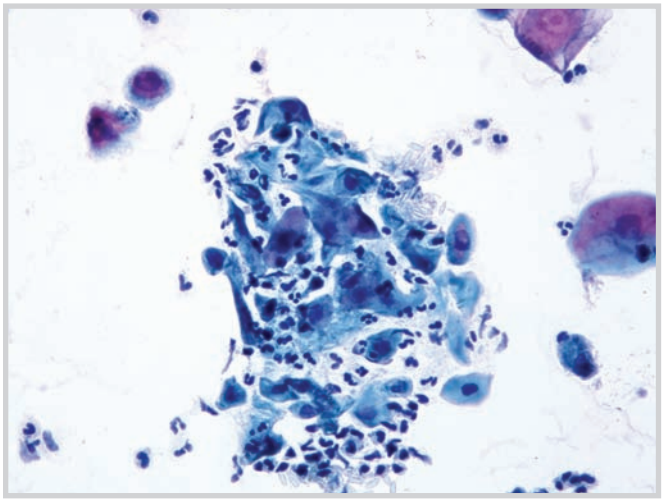


Fig. 11.9: Loose collection of histiocytes and ill-formed epithelioid cell granulomas in BCG induced changes in urine (Papanicolaou's stain X MP)

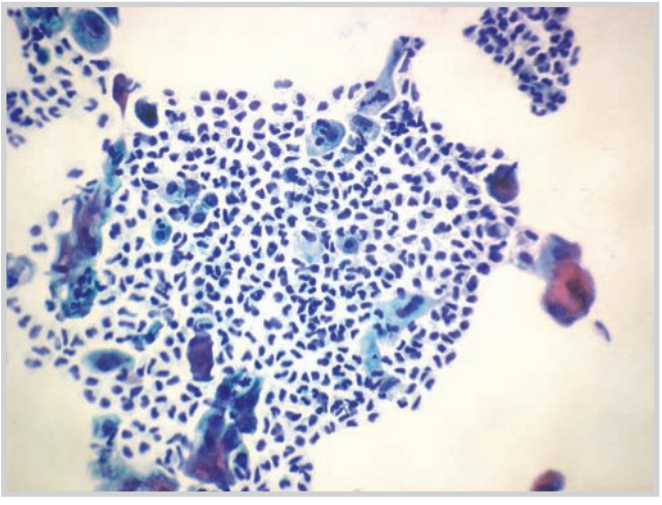


Fig. 11.10: Mild nuclear atypia in BCG induced changes in urine (Papanicolaou's stain X HP)

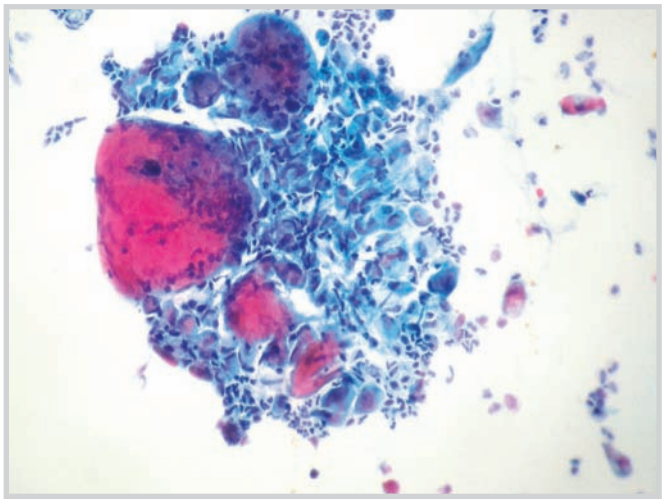


Fig. 11.11: Multinucleated cells along with polymorphs and lymphocytes in urine cytology smear in chronic radiation effect (Papanicolaou's stain X MP)

BOX 11.17 Classification of urothelial neoplasm

- Hyperplasia
  - Flat hyperplasia
  - Papillary hyperplasia
- Flat lesions with atypia
  - Reactive atypia
  - Dysplasia
  - Carcinoma in situ
- Papillary neoplasia
  - Papilloma
  - Papillary neoplasia of low malignant potential
  - Papillary carcinoma, low-grade
  - Papillary carcinoma, high-grade
- Invasive neoplasm

NEOPLASTIC LESIONS

Urothelial carcinoma of the bladder is the most common tumor of the urinary tract accounting for more than 90% of all malignant bladder tumors.[9] Urothelial carcinoma is three times more common in males than females and is more common over 50 years of age; however, it may occur in young adults and children.[10] The common environmental factors related to UC are exposure to aromatic amines used in rubber, plastic and dye, cigarette smoking, *Schistosoma hematobium* infection, long-term use of analgesics particularly phenacetin, cyclophosphamide and lithiasis.

The common presenting symptoms of the patient are hematuria, dysuria and increased urinary frequency. The urothelial neoplasms are fundamentally classified into two types: (1) flat lesions and (2) papillary lesions. Current UC is classified according to WHO and the International Society of Urologic Pathologists (ISUP) classification.[11] In this WHO/ISUP consensus classification, urothelial neoplasm is classified as mentioned in **Box 11.17**.

Basically the urothelial neoplasm can be divided into flat and papillary neoplasm. The flat lesions are classified as dysplasia and carcinoma in situ. The papillary neoplasm is divided into four groups: (1) papilloma, (2) papillary urothelial neoplasm of low malignant potential (PUNLMP), (3) low-grade papillary carcinoma and high-grade papillary carcinoma. In cytology, the papillary carcinomas are divided into low-grade papillary carcinoma and high-grade papillary carcinoma. Low-grade papillary carcinoma in cytology encompasses papilloma, PUNLMP and low-grade papillary carcinoma in WHO/ISUP classification. High-grade papillary carcinoma in cytology corresponds to that of high-grade urothelial cell carcinoma in WHO/ISUP classification.

Cytology

Low-grade Papillary Carcinoma

Low-grade papillary carcinoma is difficult to diagnose in voided urine sample (**Figs 11.12** to **11.14**).

Cytology smears of low-grade papillary UC show clusters of cells with irregular outline and multiple papillae-like structures (**Box 11.18**). This may be the only clue in the smear and therefore in the absence of a papillary structure, diagnosis may be difficult in voided urine sample. The individual cells may show mild nuclear enlargement and pleomorphism with a slight increase of the N/C ratio. Nucleoli are small and inconspicuous.

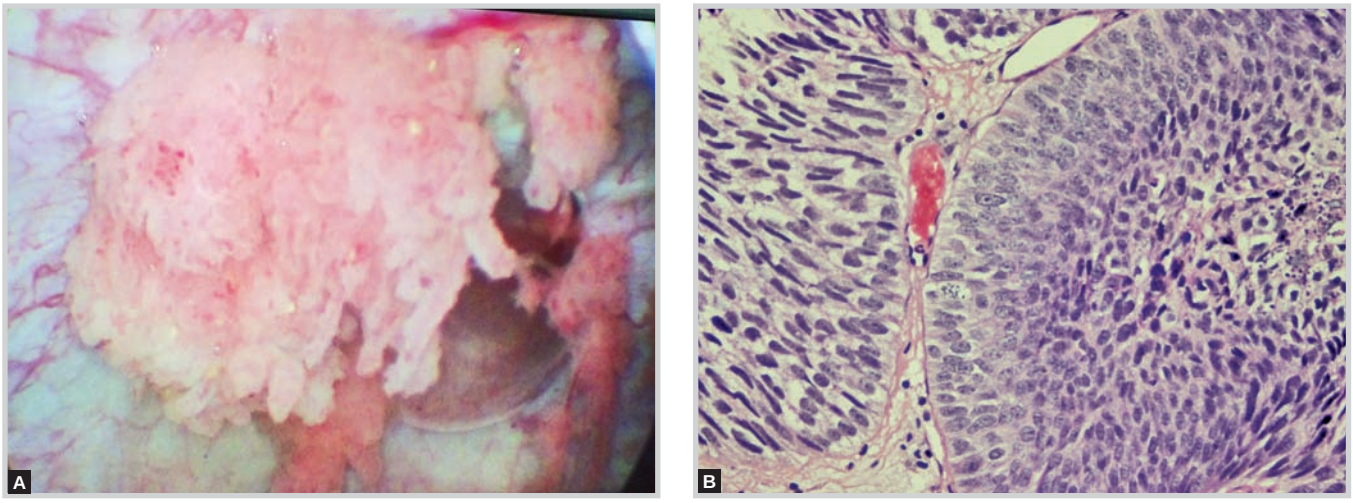
High-grade Urothelial Carcinoma and Carcinoma In Situ Lesions

Cytological features of high-grade UC are similar to that of both high-grade papillary carcinoma and carcinoma in situ of flat lesions (**Box 11.19**).

The smears show high cellularity. The cells are predominantly discrete or in loose clusters (**Figs 11.15** to **11.17**). The individual cells show moderate to marked nuclear enlargement and pleomorphism with high N/C ratio (**Fig. 11.18**). Nuclei are hyperchromatic with coarsely granular chromatin and prominent nucleoli. Nuclear membrane shows an irregular margin. Occasional cells show elongated nuclei and cytoplasmic extension resembling cercariform cells (**Fig. 11.19**). The background may show RBCs, degenerated cells and necrotic tissue fragments. The diagnosis of high-grade UC in voided sample is usually straightforward. However, if a diagnosis of high-grade UC is made but no lesion is seen in cystoscopy, then a possibility of carcinoma in situ or UC in the higher part of the urinary tract should be suggested.

Dysplasia

The term dysplasia is used in WHO classification of urothelial neoplastic lesions. In the voided urinary cytology specimen, this entity is difficult to diagnose with confidence. Moreover, the clinical significance of the diagnosis of dysplasia in the urine sample is limited as it is mostly associated with high-grade UC. It is better to avoid the diagnosis of dysplasia in urine cytology.



Figs 11.12A and B: (A) Cystoscopic picture of a low-grade papillary urothelial carcinoma of urinary bladder; (B) Histology section of low-grade transitional cell carcinoma of bladder (Hematoxylin and Eosin X MP)
Source Fig. 12 A: Dr Uttam Mete, Additional Professor, Department of Urology, PGIMER, Chandigarh, India

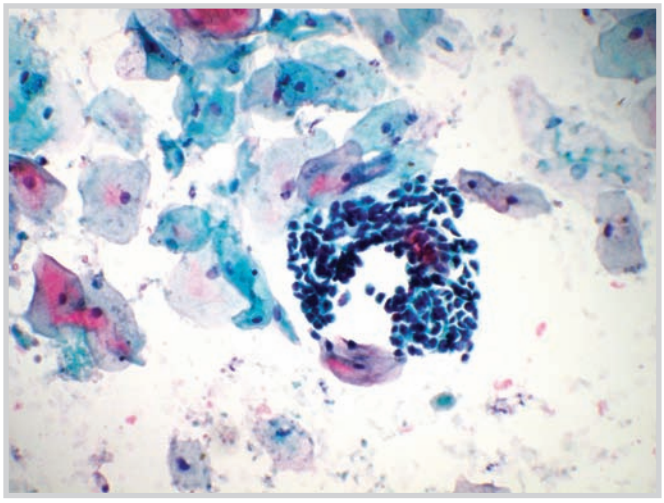


Fig. 11.13: Cytology smear of low-grade transitional cell carcinoma of bladder. The cells show scanty cytoplasm and mild nuclear enlargement (Papanicolaou's stain X MP)

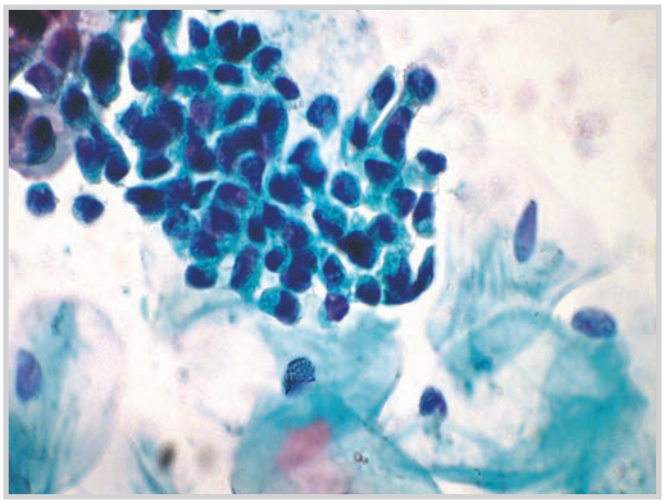


Fig. 11.14: High powered view of the same case as shown in Figure 11.13 showing scanty cytoplasm and mild nuclear enlargement (Papanicolaou's stain X HP)

BOX 11.18 **Low-grade papillary carcinoma**

- Papillary fragments of cells
- High cellularity
- Cells with enlarged nuclei
- High N/C ratio
- Monomorphic to mild pleomorphic nucleoli
- Irregular nuclear membrane
- Small inconspicuous nucleoli

BOX 11.19 **High-grade papillary carcinoma**

- High cellularity
- Dissociated and loose clusters
- Moderate pleomorphic nuclei
- Moderate to marked nuclear hyperchromasia
- High N/C ratio
- Irregular nuclear membrane
- Coarse granular chromatin
- Large prominent nucleoli
- Atypical mitosis

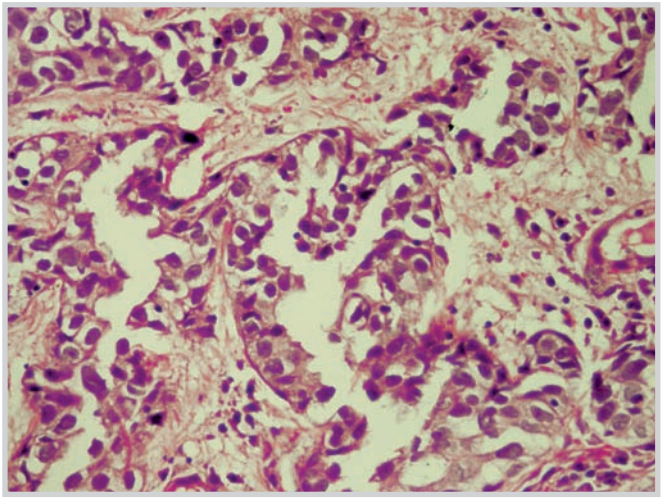


Fig. 11.15: Histology section of high-grade transitional cell carcinoma of bladder (Hematoxylin and Eosin X MP)

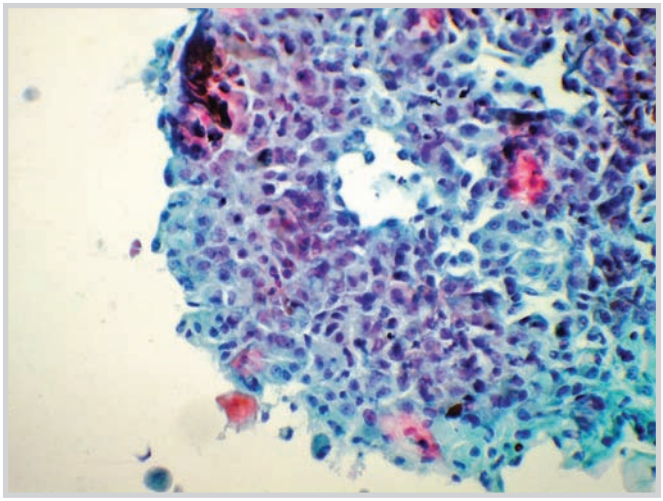


Fig. 11.16: Cytology smear of the high-grade transitional cell carcinoma of bladder showing cluster of malignant cells (Papanicolaou's stain X MP)

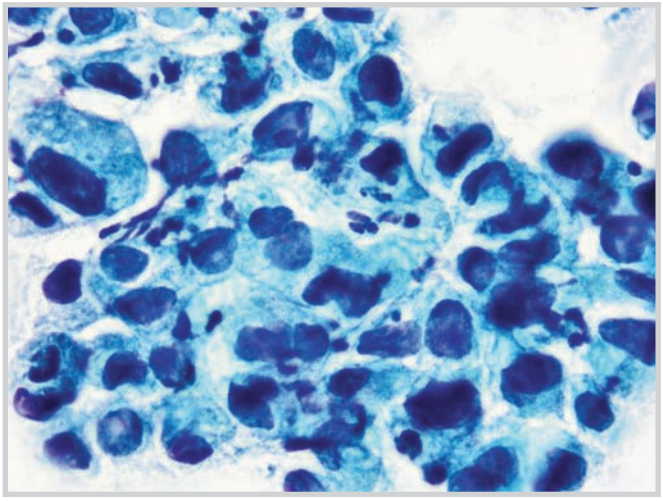


Fig. 11.17: Cytology smear of the high-grade transitional cell carcinoma of bladder showing moderate nuclear enlargement and pleomorphism (Papanicolaou's stain X HP)

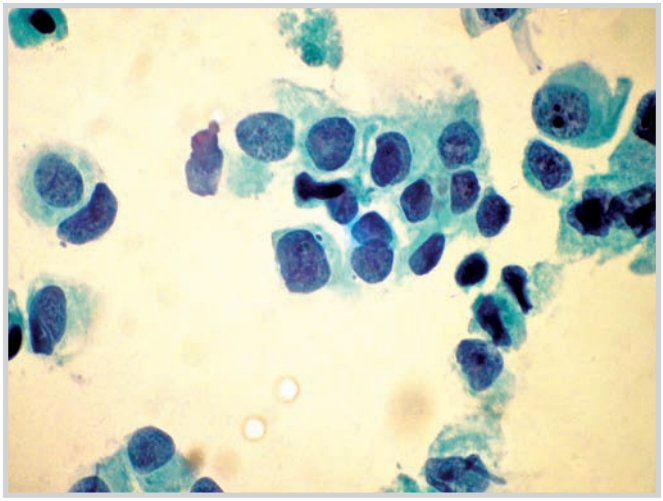


Fig. 11.18: Cytology smear of the high-grade transitional cell carcinoma of bladder showing discrete malignant cells with moderate nuclear enlargement, pleomorphism and prominent nucleoli (Papanicolaou's stain X HP)

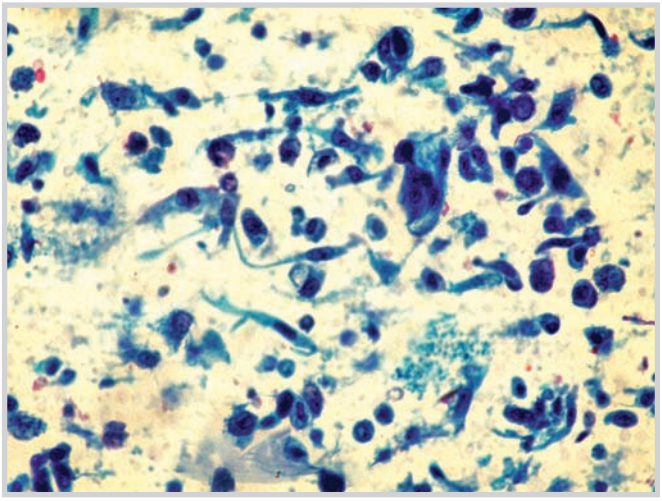


Fig. 11.19: Cercariform cells in high-grade transitional cell carcinoma of bladder (Papanicolaou's stain X HP)

Atypical Urothelial Cells

The finding of atypical urothelial cell is very common in urinary cytology smear (**Box 11.20**).

The common causes of atypical cells in urine are: viral infection, lithiasis, instrumentation, chemotherapeutic drugs and low-grade urothelial carcinoma. The cells show a mild nuclear enlargement, pleomorphism and mild hyperchromasia. The nuclear abnormalities of atypical cells are not so high to recognize them as malignant (**Figs 11.20** to **11.22**).

Approach

Clinical History: In case of the presence of atypical cells in urine, a thorough history of the patient is needed. The particular emphasis should be given on (1) the presence of viral infection, (2) history of instrumentation, (3) systematic or local history of chemotherapeutic drugs and (4) any history suggestive of urinary stone.

Repeat Urine Examination: Examination of a few more samples of urine is often helpful to ascertain the exact nature of atypical cells.

Cystoscopy: Cystoscopic examination to find out any visible lesion may help in the diagnosis. If needed, urinary brush or wash sample could be taken.

Ancillary Test: Ancillary test such as morphometric examination may be helpful to ascertain the nature of such cell.

Other Malignancies

Squamous Cell Carcinoma

Pure squamous cell carcinoma is rare in the urinary bladder. It is usually related to *Schistosoma hematobium* infection, which is more frequent near Nile river. The malignant cells show oval

BOX 11.20 Atypical cells in urine

Causes
- Infection particularly viral infection
- Lithiasis
- Instrumentation
- Chemotherapeutic drug
- Low-grade urothelial carcinoma

Cytology
- Cells with mild nuclear enlargement
- Mild hyperchromasia

Approach
- A thorough clinical history
- Repeat urine examination
- Cystoscopic examination
- Bladder wash/brush or biopsy
- Ancillary tests

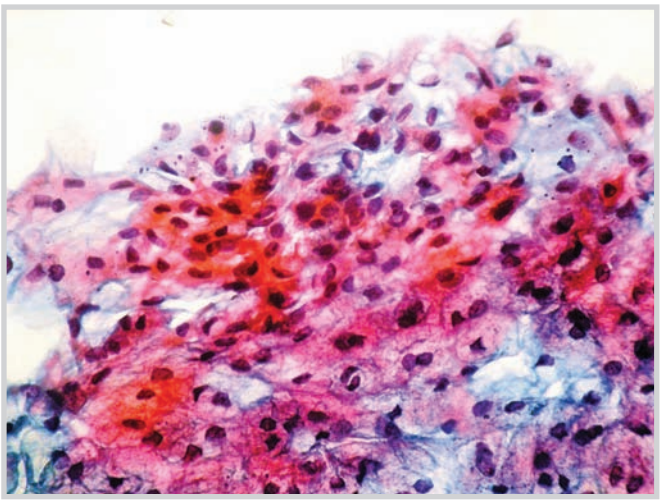


Fig. 11.21: Mild nuclear atypia in urine cytology smear (Papanicolaou's stain X MP)

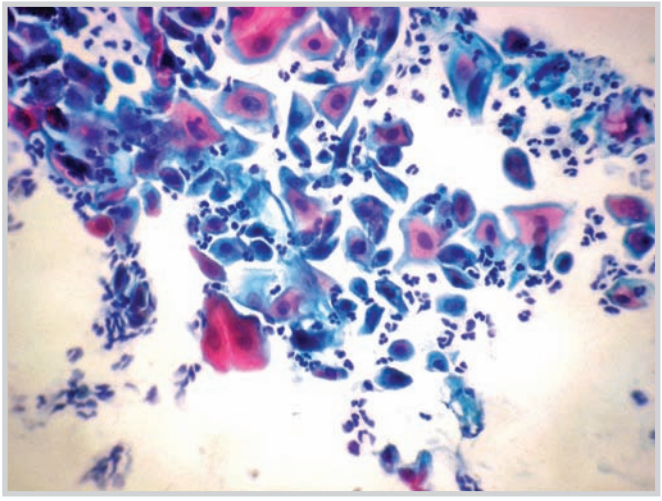


Fig. 11.20: Mild to moderate nuclear atypia in urine cytology smear (Papanicolaou's stain X MP)

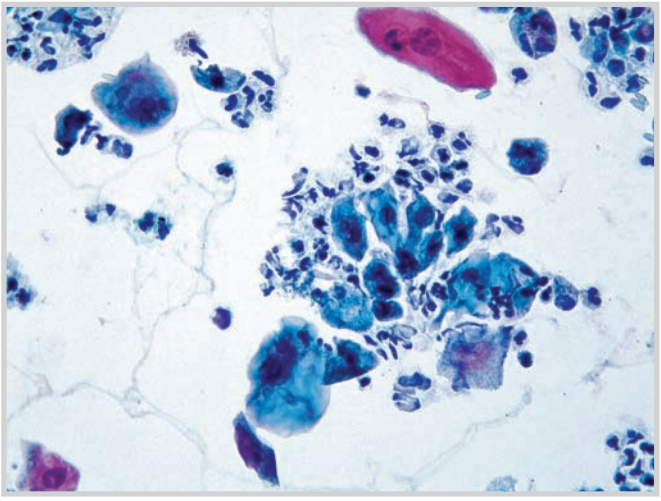


Fig. 11.22: Reactive atypia in urine cytology smear (Papanicolaou's stain X HP)

to polyhedral cells with hyperchromatic pleomorphic nuclei. The cytoplasm of the cells shows orangeophilia indicating intracellular keratinization (**Figs 11.23** and **11.24**).

Adenocarcinoma

The adenocarcinoma is rare in the urinary tract. The tumor resembles mucin secreting gastrointestinal carcinoma. The cells appear columnar in♥ appearance with pleomorphic nuclei and prominent nucleoli. Cells with glandular appearance may be noted. Abundant mucin may be present in the background.

Small Cell Carcinoma

This is a rare aggressive tumor of the bladder. Cytomorphology is similar to that of small cell carcinoma seen in other parts of the body. Smears show discrete small round cells with scanty cytoplasm (**Figs 11.25** and **11.26**). Nuclei show salt and pepper like chromatin.

Metastatic Carcinoma

Renal Cell Carcinoma

When renal cell carcinoma (RCC) invades the renal pelvis, urine cytology may show cells of RCC (**Figs 11.27** and **11.28**).[12] The individual cells show abundant clear cytoplasm and centrally placed enlarged nuclei. Urine cytology has not much value in detecting such cells as most of the cases of RCC are diagnosed by other modalities such as CT scan or MRI.

Prostatic Carcinoma

Urine cytology often shows the presence of prostate carcinoma. In most of the cases, the prior diagnosis of prostate carcinoma is known. Rarely, the primary diagnosis is made first time on

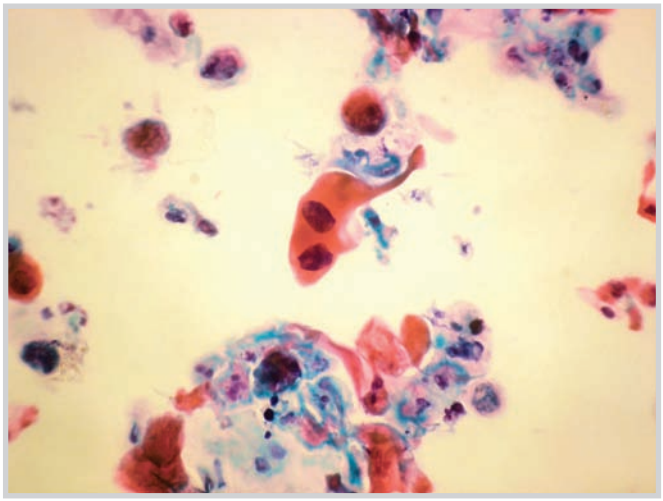


Fig. 11.23: Malignant cells with orangeophilic cytoplasm in a case of squamous cell carcinoma of bladder (Papanicolaou's stain X MP)

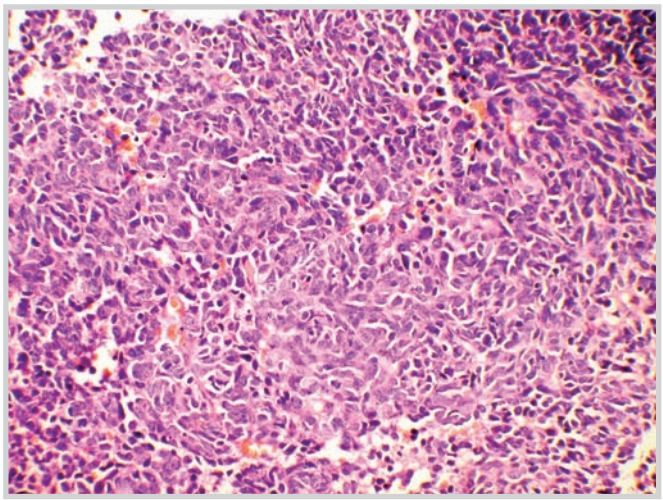


Fig. 11.25: Histology section of a small cell carcinoma of bladder (Hematoxylin and Eosin X MP)

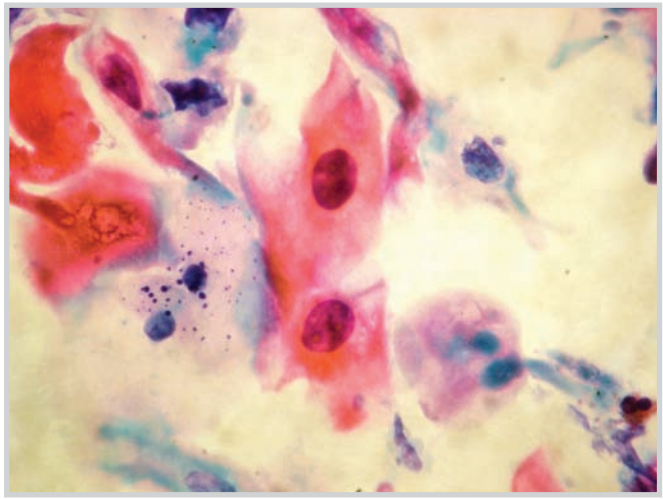


Fig. 11.24: High powered view of the squamous cell carcinoma of bladder showing polyhedral malignant cells with enlarged hyperchromatic nuclei (Papanicolaou's stain X HP)

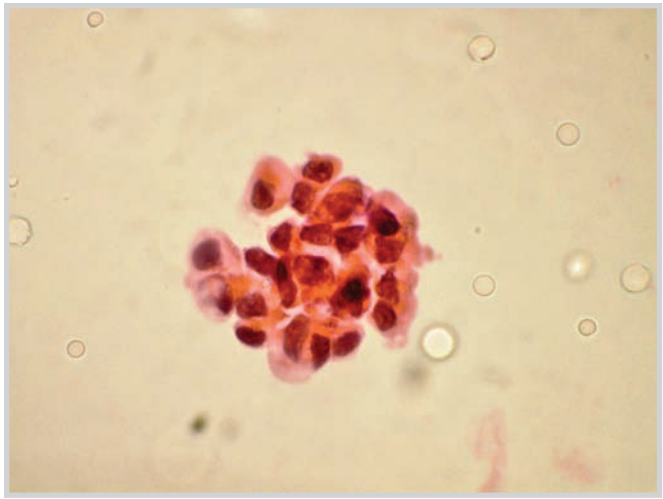


Fig. 11.26: Cluster of small cells with scanty cytoplasm in small cell carcinoma of bladder (Papanicolaou's stain X HP)

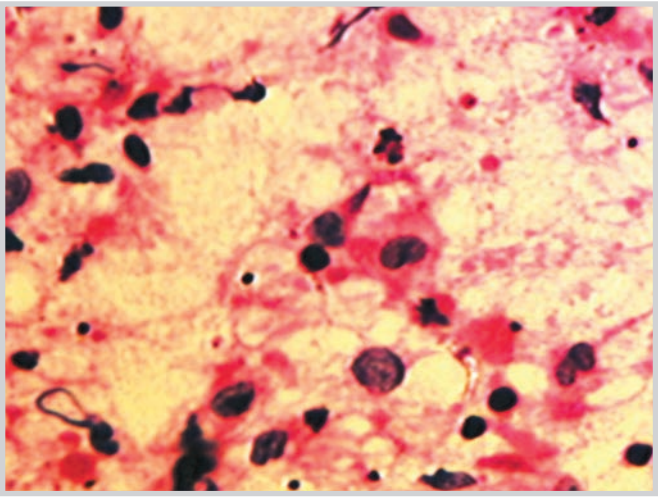


Fig. 11.27: Discrete cells with moderate amount of vacuolated cytoplasm in renal cell carcinoma (Papanicolaou's stain X HP)

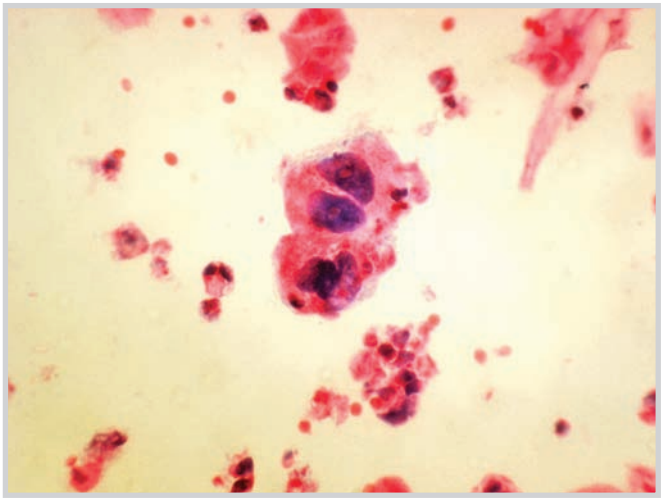


Fig. 11.28: High powered view of the same showing enlarged hyperchromatic nuclei and moderate amount of cytoplasm in renal cell carcinoma (Papanicolaou's stain X HP)

urine cytology. The individual cells show a moderate amount of cytoplasm and enlarged nuclei with prominent nucleoli. Without prior knowledge of the history of prostatic carcinoma, it is difficult to diagnose such cases.

Non-Hodgkin's Lymphoma

Primary lymphoma of the urinary bladder is very uncommon. Most of the time, bladder is secondarily involved by lymphoma. Occasionally, urine shows discrete round to oval cells with scanty cytoplasm. Nuclei show fine chromatin and single prominent nucleoli (**Figs 11.29A to C**).

Metastatic Rectal Carcinoma

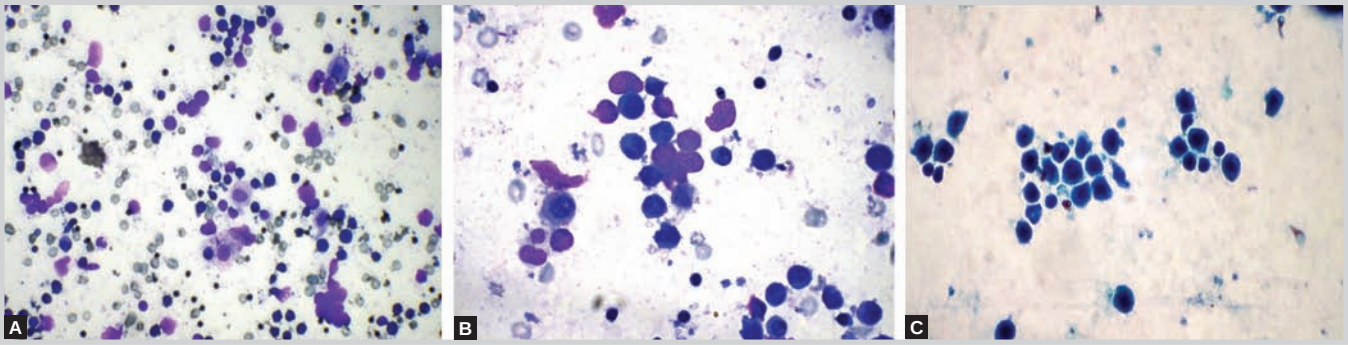
Occasionally, the urinary bladder is infiltrated by rectal adenocarcinoma. Urine cytology examination may show malignant cells. The cells are usually round to oval with moderately pleomorphic nuclei and prominent nucleoli (**Figs 11.30** and **11.31**).

Ancillary Techniques

Cytologic examination of urine is highly specific but is less sensitive technique particularly in low-grade UC. Cystoscopy is helpful for the detection of visible papillary growth; however, it is costly and invasive technique and may prove as inconclusive in cases of cystitis. Therefore, there is a need of a simple, noninvasive, highly sensitive and specific test to detect urothelial malignancy (**Box 11.21**).

Flow Cytometry

With the help of DNA flow cytometry (FCM) one can rapidly measure DNA content of a large number of cells. This can be best done in bladder wash specimen.[13] The presence of an aneuploid population or high synthetic (S) phase is considered as an indicator of malignancy. The overall sensitivity and specificity of DNA FCM are from 45% to 95% and 83% to 100%, respectively.[14-16] The sensitivity of FCM can be enhanced further by using monoclonal antibodies CK18 along with DNA FCM.[17]



Figs 11.29A to C: (A) Immature lymphoid cells in a case of non-Hodgkin's lymphoma in urine cytology (May-Grünwald-Giemsa stain X MP); (B) High powered view of the same showing immature lymphoid cells (May-Grünwald-Giemsa stain X MP); (C) Immature lymphoid cells with scanty cytoplasm (Papanicolaou's stain X HP)

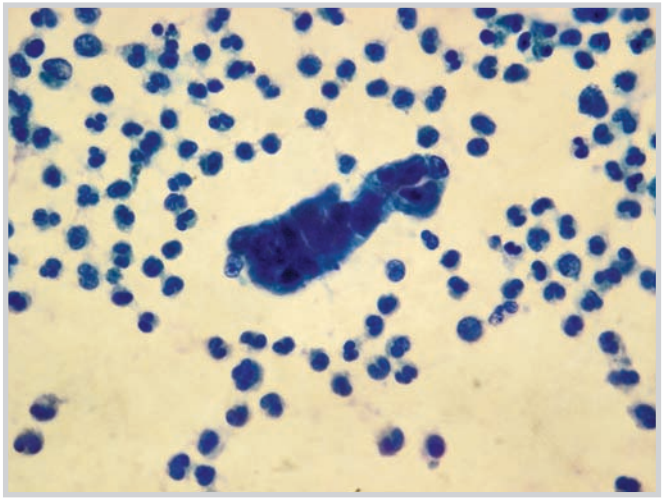


Fig. 11.30: Small cluster of malignant cells in urine in a case of rectal adenocarcinoma infiltrating into bladder (Papanicolaou's stain X MP)

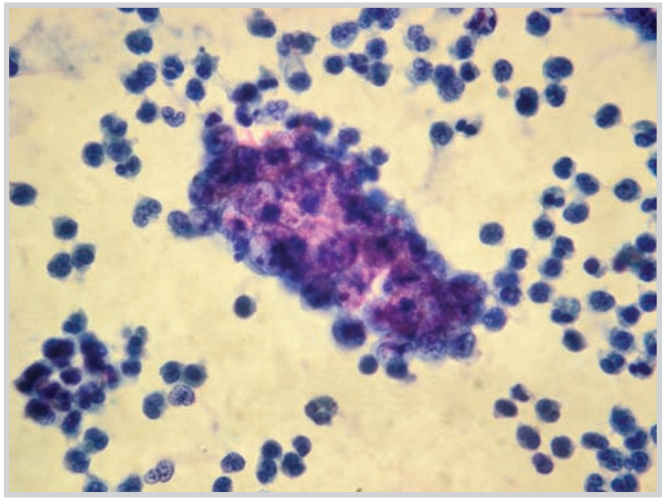


Fig. 11.31: Malignant cells with moderate nuclear enlargement and prominent nucleoli in urine in the case of rectal adenocarcinoma infiltrating into bladder (Papanicolaou's stain X HP)

BOX 11.21 **Ancillary tests to detect urothelial malignancy in urine**

- Flow cytometry
- Nuclear matrix protein-22
- Bladder tumor antigen test
- Fibrin-fibrinogen degradation product
- Hyaluronic acid and hyaluronidase
- Telomerase
- Quanticyt
- Immunocyt
- Fluorescent in situ hybridization technique
- Cytokeratin

Nuclear Matrix Protein

Nuclear matrix proteins (NMPs) are the nonchromatic structures of the nucleus that support the nuclear shape, organize DNA and play an important role in DNA replication, transcription and gene expression.[18] Nuclear matrix protein-22 is the fragmented form of the nuclear mitotic apparatus protein located in the mitotic spindle and is present at a very low level in the interphase nuclear matrix. The level of NMP-22 becomes very high in urothelial cancer.[19] The NMP-22 test is a quantitative enzyme-linked immunoassay that uses two monoclonal antibodies specific for NMP-22. Sensitivity and specificity of NMP-22 test vary from 68.5% to 88.5% and 65.2% to 91.3%, respectively.[20-23] Nuclear matrix protein-22 in urine can be quantitatively detected by bladder check test as an office-based test. This test is approved by the Food and Drug Administration in the USA.

Bladder Tumor Antigen Test

The bladder tumor antigen (BTA) is composed of basement membrane complexes that have been isolated from the urine of patients with bladder cancer. The original BTA test is a latex agglutination assay that lacks adequate specificity particularly in patients with cystitis.[24] The two other tests, BTA stat and BTA TRAK, assays measure human complement-related H factor. This factor is released from the basement membrane and its underlying matrix in patients with bladder cancer. The overall sensitivity and specificity of BTA stat are 57–83%, and 68–72% respectively, whereas, the sensitivity and specificity of BTA TRAK are 62–77% and 50–75%, respectively.[25-27] Both the above mentioned tests may show false positivity in cases of urinary tract inflammation, hematuria and urinary stone.

Fibrin Degradation Product

Fibrin degradation product (FDP) is increased significantly in urine of bladder cancer patients.[28] Accu-Dx immunoassay device can qualitatively detect FDP. It has 68% sensitivity and 86.2% specificity for detecting bladder cancer.[29]

Hyaluronic Acid and Hyaluronidase

Hyaluronic acid (HA), a nonsulfated glycosaminoglycan, is elevated in urothelial cancer.[30] The overall sensitivity and specificity of HA tests are 83% and 90% for detecting bladder cancer regardless of tumor grade.[31]

Telomerase

Telomerase is a specific ribonucleoprotein that can be detected by specific telomerase repeat amplification protocol. The human telomerase terminal transferase enzyme is a ribonucleoprotein that synthesizes TTAGGG repeats. Increased telomerase activity has been documented in several neoplastic conditions including urothelial cancer.[32] The sensitivity and specificity of telomerase activity in the urine sample for detecting bladder cancer are 70–86% and 60–90%, respectively.[33,34] The contaminated urine sample may produce false negative results. The false positive result may be noted in inflammation.

Quanticyt

Quanticyt system applies the image morphometric analysis and DNA content of the cell to detect urothelial cancer.[35] This laboratory-based test shows 59–69% sensitivity and 70% specificity for detecting bladder cancer.[35] This technique requires bladder wash for optimal cellularity and technical expertise.

Immunocyt

Immunocyt test is the combination of cytology with an immunofluorescence assay that detects cell markers specific for urothelial cancer. This test is 86% sensitive and 79.4% specific for detection of bladder cancer.[36]

Fluorescent In Situ Hybridization Technique

Interphase fluorescent in situ hybridization uses DNA probes to chromosomal centromeres or unique loci to detect cells with numerical or structural abnormalities as seen in urothelial cancer.

Loss of whole chromosome 9 or part of it is the most common genetic alteration in bladder carcinoma. There may also be frequent alterations of chromosomes 17, 7, 11 and 1 in urothelial cancer. Fluorescent in situ hybridization in urine sample shows 81% sensitivity and 98% specificity.[37,38]

Cytokeratin

Cytokeratin-20 (CK-20) is expressed in urothelial carcinomas but not in normal urothelial cells. Therefore measurement of CK level in urine may be helpful to screen UC.[39] Sensitivity and specificity of CK-20 in urine sample are 91% and 67–100%, respectively.[40]

Table 11.1 shows sensitivity and specificity of different ancillary techniques used in urine specimen to detect malignancy.

DIAGNOSTIC ACCURACY OF URINE CYTOLOGY

Sensitivity and specificity of urine cytological examination are 40–62% and 94–100%, respectively.[41-43] Diagnostic sensitivity and specificity of the urine cytological examination depends on several factors such as:

- *Specimen type*: Whether voided urine or bladder wash/brush. Bladder wash/brush usually yields better cellularity and diagnostic material.[44]
- *Diagnostic category used*: Diagnostic criteria used in urinary cytology report influences the accuracy of the urinary cytological examination.[45]
- *Grade of urothelial carcinoma*: Low-grade UC has low sensitivity and specificity. These cases are difficult to diagnose in urinary cytology as the cytological features are subtle and difficult to find out, whereas high-grade UC has high sensitivity and specificity.
- *Screening or symptomatic patient*: Whether urinary examination is done as screening or in symptomatic patient. Sensitivity of urine cytology is much higher if the test is used in screening of symptomatic patients.

The false positive rate in urine cytology varies from 1% to 15%. The predominant causes of false positive urinary cytology are lithiasis, infection and instrumentation (**Box 11.22**).

TABLE 11.1: Comparison of different ancillary tests in urine sample

Test	Specific marker	Basic test type	Sensitivity (%)	Specificity (%)
Flow cytometry	Aneuploid cell and high S-phase	Flow cytometry	45–95	83–100
NMP-22	Nuclear matrix protein: nuclear mitotic apparatus	Enzyme-linked immunoassay	68–88	65–91
Bladder tumor antigen test	H factor of complement	Immunoassay	62–77	68–72
Accu-dx	Fibrinogen degradation product	Immunoassay	68	86
Hyaluronic acid and hyaluronidase	Hyaluronic acid Hyaluronidase	Immunoassay	83	90
Telomerase	RNA polymerase	Telomeric repeat action protocol	70–86	90
Quanticyt	Nuclear morphometry and DNA	Image morphometry	59–69	70
Immunocyt	CEA and mucin	Immunofluorescence	86	79
Fluorescent in situ hybridization technique	Chromosomal aberration	In situ hybridization	81	98

BOX 11.22 Causes of false positive urinary cytology

- Viral cystitis
- Lithiasis
- Instrumentation: Catheterization, etc.
- Chemotherapeutic drugs: Cyclophosphamide, busulfan, BCG vaccine, etc.
- Radiation therapy
- Ileal conduit specimen
- Ill-preserved degenerated cell
- Inflammation causing atypia

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CHAPTER 12

Respiratory Cytology

Chapter Contents

NORMAL ANATOMY AND HISTOLOGY

The respiratory system is broadly divided into the upper respiratory tract, lower respiratory tract and lungs (**Fig. 12.1**).

Upper respiratory tract: It extends from nose to larynx and is comprised of nose, paranasal sinuses, pharynx and larynx.

Lower respiratory tract: It extends from the trachea to the lungs and is comprised of trachea, bronchi and bronchioles

Lungs: It is made of respiratory bronchioles, alveolar ducts, alveolar sacs and alveoli.

Histology

The upper respiratory tract is lined by stratified squamous epithelium and ciliated columnar epithelial cells.

Table 12.1 shows mucosal lining of the epithelium from the nose to the alveoli. Most of the portion of the respiratory tract from the nose to the bronchi is lined by ciliated columnar epithelium except vestibules of the nose, vocal cords and superior surfaces of the epiglottis, which are lined by non-keratinizing stratified squamous epithelium.

Figure 12.2 shows histology of the lung alveoli. The alveolar epithelium of the lung is lined by Type I and Type II

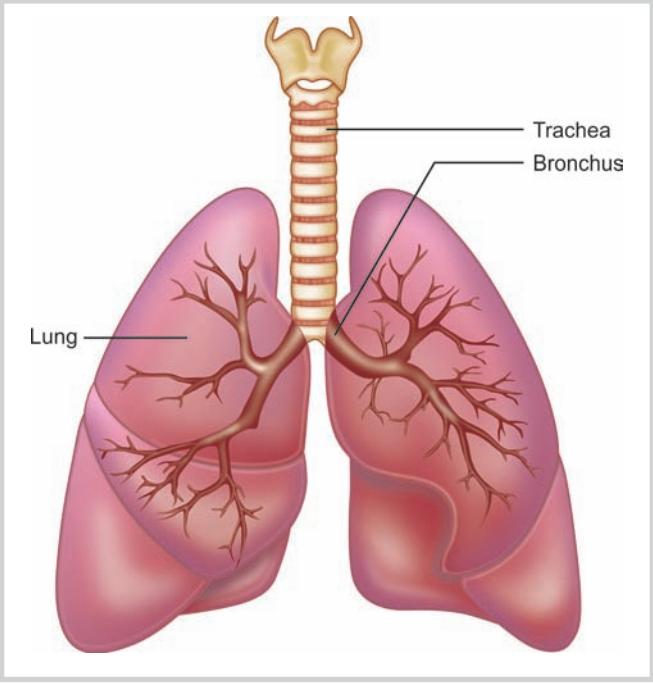

Fig. 12.1: Schematic diagram shows anatomy of the respiratory tract

TABLE 12.1: Lining of respiratory tract

Parts	*Mucosal lining*
Nasal vestibules	Stratified squamous epithelium
Nasal sinuses	Ciliated columnar epithelium
Nasopharynx	Ciliated columnar epithelium
Larynx	Mostly : Ciliated columnar epithelium Vocal cords and superior surfaces of the epiglottis: Stratified squamous epithelium
Trachea and bronchi	Ciliated columnar epithelium, goblet cells, neuroendocrine cells
Terminal bronchioles	Simple columnar to cuboidal epithelium
Alveoli	Type I and II pneumocytes and alveolar macrophages

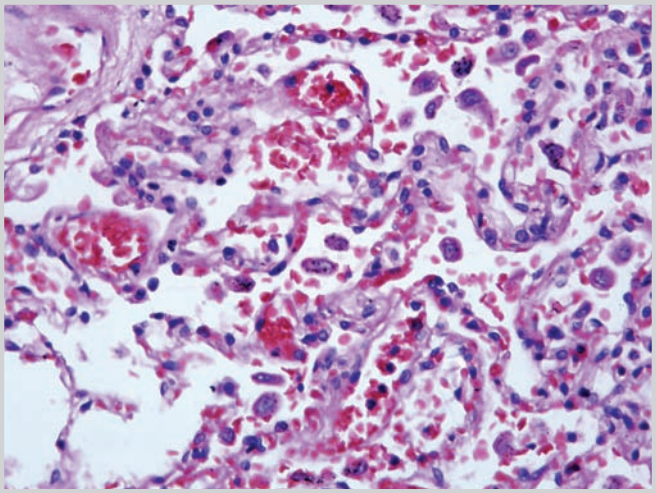

Fig. 12.2: Histology section of the lung showing normal alveoli (Hematoxylin and Eosin X MP)

pneumocytes. Type I pneumocytes cover more than 95% of the alveolar surface. The cells are long, thin and flattened. Type II pneumocytes cover less than 5% of the alveolar surface. The cells are round with vacuolated cytoplasm and centrally placed nuclei having prominent nucleoli. Electron microscopy of the Type II pneumocytes shows membrane-bound lamellar bodies that contain pulmonary surfactant. Alveolar macrophages are large cells with abundant vacuolated cytoplasm that contain phagocytosed material. Nuclei are central in position and round monomorphic in shape.

SAMPLING TECHNIQUES

Detailed knowledge of the sampling and preparation of respiratory specimen is very essential because the diagnostic accuracy depends vastly on sampling method. The commonly used sampling techniques are: sputum, bronchial wash and brush, bronchoalveolar lavage (BAL), transbronchial needle aspiration (TBNA) and ultrasound-guided fine needle aspiration cytology (USG FNAC).

BOX 12.1 **Sputum**

Early morning deep coughed sputum
Fresh sample in clean wide bore container
Advantages
- Easy to collect
- Wide area of respiratory tract is covered
- Simple and noninvasive
- Good sensitivity for central tumor
- Best for screening malignancy

Disadvantages
- Exact localization of the tumor not possible
- Considerable degeneration of the cells
- Not good for peripheral tumors

Sputum

Sputum (**Box 12.1**) is the most commonly examined respiratory sample. Sputum is easy to collect and easy to examine. Early morning deep coughed sputum should be collected in a clean container. If the patient is unable to produce sputum then expectoration can be induced by inhalation of stream from saline water. Consecutive three morning sputum samples are recommended for better yield of cells and to increase diagnostic sensitivity. Examination of fresh sputum sample is recommended. However, if this is not possible then the sputum sample can be collected in 70% ethyl alcohol solution for fixation.

Sputum sample should be kept in a petri dish on white background. Any tissue fragment or blood-mixed material is taken and multiple smears are made. Both alcohol-fixed and air-dried smears should be kept for staining.

Bronchial Specimens

Bronchial Aspirate and Wash

Introduction of flexible fiberoptic bronchoscope enables the clinician to procure samples from the peripheral part of the lower respiratory tract. With the help of bronchoscope, the bronchial secretion is aspirated directly. In case of bronchial washing technique, 3–5 mL of balanced salt solution or normal saline is introduced through the bronchoscope and the fluid is re-aspirated. The direct smear or multiple smears from the centrifuged fluid sample are made for staining.

Bronchial Brush

The lesion is directly visualized by the fiberoptic bronchoscope. The brush is applied to the lesion through the instrument. Multiple smears are made and fixed in 95% ethanol or the brush is rinsed in a collection solution and sent to the laboratory. The main advantage of the brush is that direct visualization of the lesion is possible and therefore diagnostic cellular yield is excellent.

BOX 12.2 Bronchoalveolar lavage

Procedure
- Repeatedly (five times) 20 mL of normal saline is flushed through bronchoscope in the desired segment of the lung
- Gentle suction is done to collect the lavage specimen

Indications
- Nonresolving pneumonia
- Interstitial lung disease
- To diagnose opportunistic infection in an immunocompromised host
- Suspected case of peripheral lung carcinoma

Contraindications
- Severe coughing
- Dyspnea
- Cardiac arrhythmias

Adequacy of the sample
- Cell count more than 2 X 10^6
- More than 10 macrophages per high power field

Unsatisfactory sample
- Excessive blood
- Cellular degeneration
- Predominant squamous cells
- Artifacts changes

Bronchoalveolar Lavage (Box 12.2)

Bronchoalveolar lavage (BAL) is a powerful investigative technique in the field of pulmonary medicine.

Procedure: Bronchoalveolar lavage should be done before performing any other techniques such as bronchial wash/brush or biopsy. Under local anesthesia, fiberoptic bronchoscope is wedged in a desired subsegmental bronchus at the desired location. At first, 20 mL of normal saline is flushed through bronchoscope in the desired segment of the lung and gentle suction is done to collect the lavage specimen. The whole procedure is done for five times to get 40–50 mL solution. Bronchoalveolar lavage specimen should be processed as early as possible.

Indications: The predominant clinical indications of BAL are:
- Any case of nonresolving pneumonia
- Interstitial lung disease
- To diagnose opportunistic infection in an immunoco-mpromised host
- Suspected case of peripheral lung carcinoma

Bronchoalveolar lavage is particularly used for the diagnosis of *Pneumocystis carini* infection. Other than diagnosis, BAL is also useful for therapy of alveolar proteinosis, cystic fibrosis and pulmonary alveolar microlithiasis.[1]

Contraindications

Bronchoalveolar lavage is contraindicated in:
- Severe coughing
- Dyspnea
- Cardiac arrhythmias

Ideally, BAL should be avoided in severe respiratory failure.

Adequacy of BAL Sample

There are no absolute indicators of adequacy of the BAL sample. However, it is considered that cell counts more than 2 X 10^6, or more than 10 macrophages per high power field are the markers of adequacy of the sample.[2] The BAL sample is labeled unsatisfactory when it shows excessive blood, degeneration and squamous cells.

Transbronchial Needle Aspiration

Transbronchial needle aspiration is done in those cases where the lesion is located in the subbronchial region. It is done through the fiberoptic bronchoscope, and multiple air-dried and alcohol-fixed smears are made. TBNA avoids other surgical procedures such as mediastinoscopy or surgical biopsy. Sensitivity of TBNA alone is near about 56% and specificity is 74%.

Transesophageal Fine Needle Aspiration

Fine needle aspiration cytology can be done by esophageal endoscopy and passing the needle through the esophagus. Ultrasound guidance in addition of the esophagoscope may improve the diagnostic accuracy of the technique. Transesophageal fine needle aspiration is particularly helpful in sampling of mediastinal lymph node.

Percutaneous Fine Needle Aspiration Cytology

Percutaneous fine needle aspiration cytology is an effective technique for rapid diagnosis of pulmonary mass lesions. It is usually done under USG or CT scan guidance with the help of 22 gauge needle. The sensitivity and specificity of percutaneous FNAC are 89% and 96%, respectively.[3]

Table 12.2 compares the different sampling techniques in the respiratory system.

NORMAL CYTOLOGY

The normal cellular components of the respiratory tract are squamous epithelial cells, ciliated columnar cells, alveolar macrophages and goblet cells (**Box 12.3**).

Squamous Epithelial Cells

The squamous cells come from the oral cavity as contaminants and are predominantly seen in sputum samples. The cells are superficial squamous cells and many of them are aneucleated.

Columnar Cells

The ciliated columnar cells (**Box 12.4**) are the predominant lining cells of the lower respiratory tract. These cells are usually not seen in the sputum samples. They are mainly noted in bronchial

TABLE 12.2: Comparison of different sampling techniques of respiratory system

Techniques	*Procedure*	*Advantages*	*Disadvantages*
Sputum	Early morning sputum is collected in a container	• Easy to collect • The wider area of the respiratory tract is covered • Simple and noninvasive • Good sensitivity for central tumor • Best for screening malignancy	• Exact localization of the tumor not possible • Degeneration of the cells • Not good for peripheral tumors
Bronchial wash	Balanced salt solution or normal saline is introduced through the bronchoscope and the fluid is re-aspirated	• Cellularity more than sputum • Morphology is better preserved	• Better for central lesion • Lesion is not well visualized
Bronchial brush	The brush is applied on the lesion through the bronchoscope and multiple smears are made	• Direct visualization of the lesion is possible • Morphology is well preserved • Much more sensitive than sputum or wash	• Sophisticated technique • Suitable only for central localized lesion
Bronchoalveolar lavage	Normal saline is flushed through bronchoscope in the desired segment of the lung and gentle suction is done to collect the lavage specimen	• Useful to diagnose peripheral lung lesions such as infection, alveolar diseases • High sensitivity in diagnosing bronchoalveolar carcinoma	Technical skill is needed
TBNA	It is done through the fiberoptic bronchoscope	• Good for sub-bronchial lesion • Better morphology	• Swelling is needed • Sophisticated technique

Abbreviation: TBNA—transbronchial needle aspiration

BOX 12.3 Normal constituents of lung samples

Lining cells
- Squamous epithelial cells
- Ciliated columnar cells
- Alveolar macrophages
- Goblet cells

Associated cells
- Polymorphs
- Lymphocytes
- Histiocytes

Acellular material
- Curschmann's spirals
- Ferruginous (asbestos) bodies
- Charcot-Leyden crystals
- Carbon particles
- Hemosiderin

Contaminants
- Pollen grains
- Vegetable materials
- Contaminants from food particles

BOX 12.4 Ciliated columnar cell

- Present singly and also in honeycomb-like clusters
- Columnar-shaped
- Terminal bar with cilia in one end
- Nuclei present basally
- Round, regular monomorphic nuclei
- Inconspicuous nucleoli
- Nuclear chromatin fine

washing/brushing and BAL specimens. The cells are columnar in appearance with moderate cytoplasm. Nuclei are located on one side of the cell. Cilia come out from the terminal plate of the columnar cells (**Fig. 12.3**).

Goblet Cells

Goblet cells (**Box 12.5**) are present mainly in bronchial washing/brushing or BAL samples and rarely seen in sputum. The individual cells have a moderate amount of vacuolated cytoplasm filled with mucin (**Fig. 12.4**). The nuclei are round and monomorphic. Goblet cells are seen in abundant numbers in chronic tracheobronchial irritation and asthma.

Alveolar Macrophages

Alveolar macrophages (**Box 12.6**) are the "key cells" that indicate adequacy of sputum sample and in their absence, the sputum sample has not much diagnostic value. The alveolar macrophages show moderate to abundant cytoplasm filled with phagocytosed brownish to blackish dust particles (**Fig. 12.5**). The nuclei are

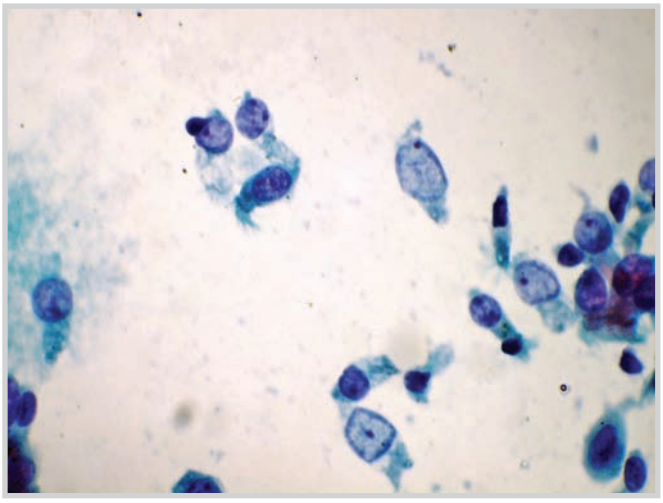

Fig. 12.3: Ciliated columnar cell of normal lining cell of the bronchus. Cilia arise from the terminal plate of the cell (Papanicolaou's stain X HP)

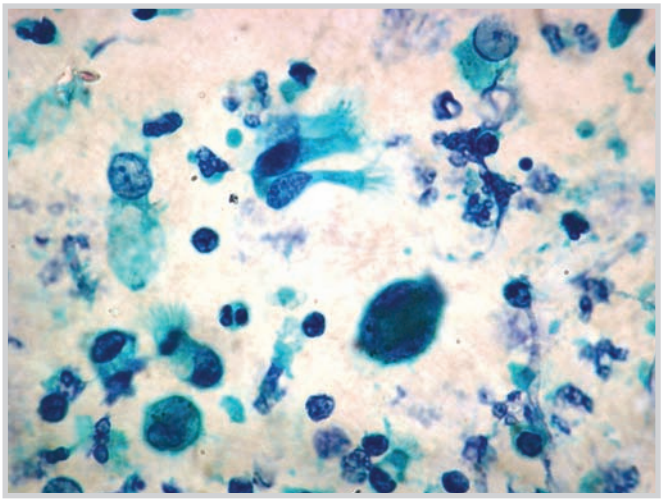

Fig. 12.5: The alveolar macrophages show brownish to blackish dust particles in the cytoplasm (Papanicolaou's stain X HP)

BOX 12.5 Goblet cells

- Present in small clusters
- Cells with moderate amount of vacuolated cytoplasm
- Round, monomorphic nuclei
- Abundant goblet cells seen in bronchial asthma, chronic bronchitis

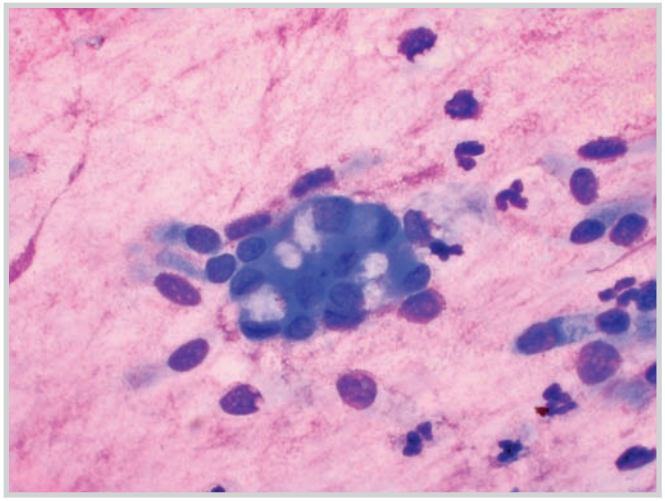

Fig. 12.4: Small cluster of goblet cells with vacuolated cytoplasm (May-Grunwald Giemsa stain X HP)

centrally placed with convoluted kidney-shaped. Binucleation and multinucleation are common in alveolar macrophages.

Other Cells

In the absence of inflammation, scattered occasional polymorphs and lymphocytes are also seen in respiratory sample.

BOX 12.6 Alveolar macrophages

- Indicate adequacy of sputum sample
- Always present singly
- Round cells with abundant cytoplasm
- Cytoplasm filled with brownish to blackish dust particles
- Centrally placed kidney-shaped nuclei
- Bi- and multinucleation present
- Fine chromatin

NONCELLULAR COMPONENTS (Table 12.3)

Mucus

These are acellular homogenous substances. The largest amount of mucus is usually present in bronchial obstruction and bronchioloalveolar carcinoma.

Curschmann's Spirals

Curschmann's Spirals are corkscrew-shaped long inspissated mucus cast produced in the small bronchioles due to excessive mucus production. These are commonly seen in condition with excessive mucus production, such as chronic bronchitis, asthma and in smokers.

Ferruginous Bodies

The ferruginous (asbestos) bodies are approximately 50–200 micron long rod-shaped golden-brown structures with two terminal bulbous tips. These are usually asbestos fibers coated with protein and iron. The presence of ferruginous bodies in the sputum or BAL usually indicates asbestos exposure.

TABLE 12.3: Noncellular components in respiratory sample: morphology and significance

Material	*Morphology*	*Significance*
Curschmann's Spirals	Corkscrew-shaped long inspissated mucus cast	Present in chronic bronchitis, asthma and in smokers
Ferruginous (asbestos) bodies	Long rod-shaped golden-brown structures with two terminal bulbous tips	Present in asbestos exposure
Charcot-Leyden crystals	Bi-pyramidal needle-shaped crystal with two pointed ends	Present in asthma patients, eosinophilic pneumonia and allergic bronchopulmonary aspergillosis
Mucus	Acellular homogenous substances	Bronchial obstruction and bronchioloalveolar carcinoma
Psammoma body	Darkly stained concentric lamellated structures	Present in bronchioloalveolar carcinoma

Charcot-Leyden Crystals

These are bi pyramidal needle-shaped crystals with two pointed ends. These crystals are composed of granules of eosinophils. Charcot-Leyden crystals are seen frequently in asthma patients, eosinophilic pneumonia and allergic bronchopulmonary Aspergillosis.

Psammoma Body

These are darkly stained concentric lamellated structures made up of calcium. Psammoma bodies are usually seen in bronchioloalveolar carcinoma.

Others

In addition, respiratory specimen may contain pollen grains, vegetable materials or contaminants from food particles (**Fig. 12.6**).

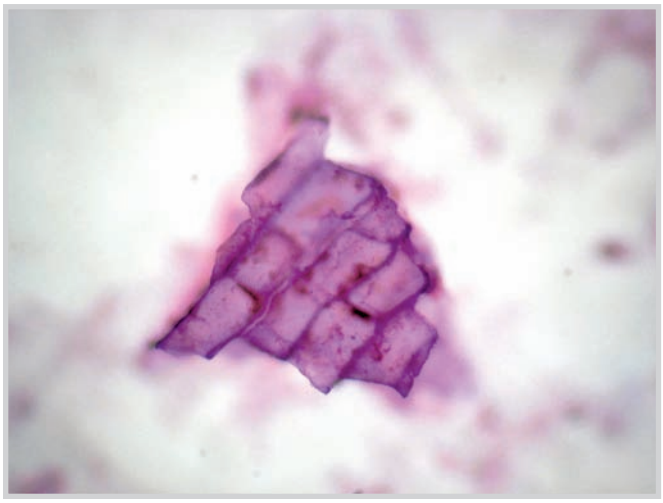

Fig. 12.6: Vegetable material in the sputum (Papanicolaou's stain X HP)

BENIGN CELLULAR ABNORMALITIES

Various non-neoplastic lesions may affect different components of the respiratory epithelial cells such as:

- Squamous cells
- Bronchial epithelium
- Reserve cell hyperplasia
- Hyperplasia of type II pneumocytes

Abnormalities of Squamous Cells

Inflammatory Changes

The inflammatory changes of the squamous epithelial cells are very common. Nuclear atypia is seen in infection of lung, trauma or infection. The cells show a mild nuclear enlargement, coarse chromatin and hyperchomasia. Nuclei of these cells often show degenerative changes. These cells should not be misinterpreted as cancer. In contrast to malignant cells, the cells show regular nuclear membrane and monomorphic nuclei.

Papanicolaou Cells

Papanicolaou (PAP) cells are small squamous cells with round hyperchromatic nuclei. The cells are related to inflammation of lower respiratory tract. These atypical cells should not be mistaken for cancer cells because of hyperchromasia and enlarged nuclei. Unlike cancer cells, they have regular monomorphic nuclei with regular nuclear margin. George Papanicolaou noted these atypical cells in his sputum after respiratory tract infection and the cells are named after his name.[4]

Abnormalities of Bronchial Epithelial Cells

Ciliocytophoria

There is a very important point about cilia in respiratory epithelial cells. The cells in the presence of cilia degenerate and may show only terminal plate. Infection particularly viral infection of the bronchial lining epithelial cells may cause detachment of cilia from the cell. This condition is known as ciliocytophoria. The cytology smear shows detached fragment of cilia or cells with cytoplasm and nucleus without any cilia. There is no association of malignancy with ciliocytophoria.

BOX 12.7 **Bronchial cell hyperplasia (Creola body)**

Commonly seen
- Chronic bronchitis
- Tuberculosis, bronchial
- Asthma

Morphology
- Large three-dimensional papillary clusters of the bronchial cells
- Smooth peripheral outline
- Focal areas of the surface cells show cilia
- Outer cells in palisade arrangement
- Cells are small with monomorphic nuclei
- Fine nuclear chromatin
- Occasional prominent nucleoli

Differential diagnosis: Adenocarcinoma

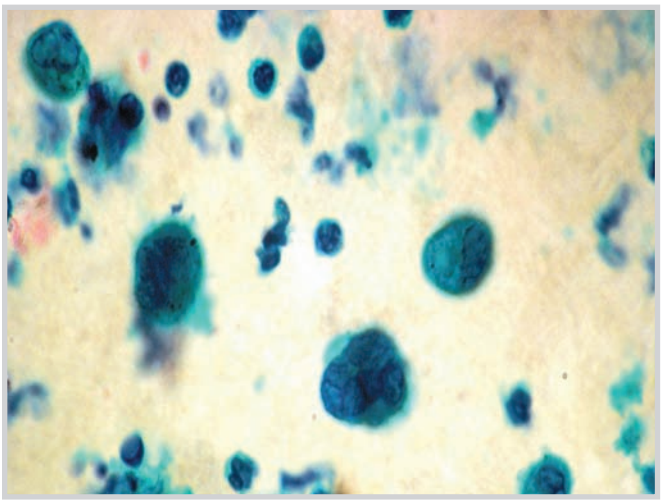

Fig. 12.8: Frequent multinucleation in the reactive bronchial hyperplasia (Papanicolaou's stain X HP)

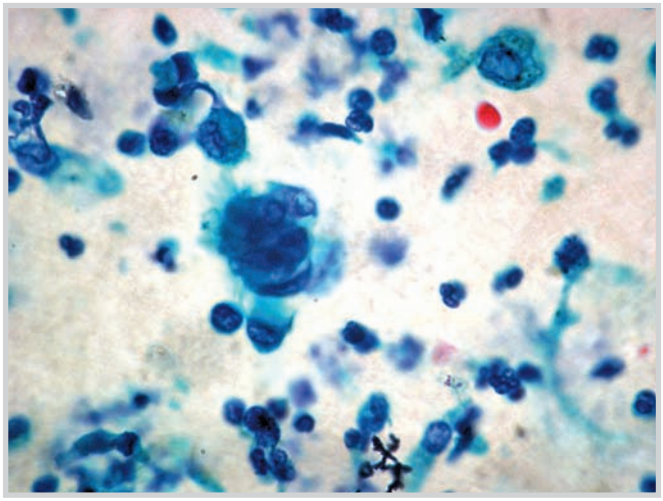

Fig. 12.7: Nuclear enlargement and pleomorphism in the reactive bronchial hyperplasia (Papanicolaou's stain X HP)

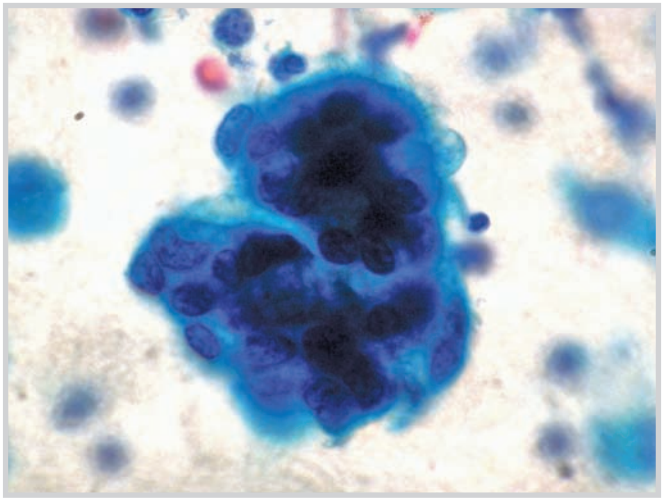

Fig. 12.9: Large three-dimensional papillary clusters of the bronchial cells with smooth peripheral outline in hyperplastic bronchial epithelial cells (Papanicolaou's stain X HP)

Hyperplastic Bronchial Epithelium

The chronic irritation or infection, such as chronic bronchitis, tuberculosis, bronchial asthma, etc. may cause hyperplasia of the bronchial epithelial cells (**Box 12.7**). The bronchial cells show variable degree of nuclear enlargement and pleomorphism (**Fig. 12.7**). The cells may show prominent nucleoli and clumped chromatin. Bi- and multinucleated cells may also be seen (**Fig. 12.8**). The hyperplastic bronchial epithelial cells show large three-dimensional papillary clusters of the bronchial cells with smooth peripheral outline (**Fig. 12.9**). The outer layer of the cell is often arranged in palisade manner. The cells on the surface of the clusters often show cilia. The cells are small with monomorphic nuclei, fine nuclear chromatin and occasional prominent nucleoli. This cluster of cells is also called as Creola body. The name was kept by the name of the patient in whom it was first recognized. The Creola body may be mistaken for adenocarcinoma due to the papillary arrangement of the cells. However, individual cells of the cluster do not show any evidence of malignancy.

Bronchial Reserve Cell Hyperplasia (Box 12.8)

During lung injury of infection, the superficial bronchial epithelial cells may be damaged and the basal layer of cells may proliferate.

The hyperplastic basal cells are usually arranged in small tight clusters. The cells are small with scanty cytoplasm and round mildly hyperchromatic nuclei. Nucleoli are usually absent but may show prominent nucleoli. Nuclear molding is also noted at times.

The cells may often resemble small cell carcinoma because of small size, hyperchromatic nuclei and nuclear molding.

BOX 12.8 Reserve cell hyperplasia

Causes: Infection, injury
Morphology
- Tight cohesive small clusters
- Small cells
- High N/C ratio
- Scanty cytoplasm
- Hyperchromatic nuclei
- Nuclear molding

D/D: Small cell carcinoma

BOX 12.9 Hyperplastic type II pneumocytes

Causes: Tuberculosis, pulmonary infraction, radiation injury, chemotherapeutic drugs, pneumonia, interstitial lung disease
Morphology
- Tight papillary clusters
- Abundant vacuolated cytoplasm
- Enlarged nuclei
- Fine chromatin
- Prominent nucleoli

D/D: Adenocarcinoma

However, the basal cells are arranged most of the time in tight clusters and the nuclei have prominent nucleoli.

Hyperplasia of Type II Pneumocytes

Hyperplasia of Type II pneumocytes (**Box 12.9**) is seen in various conditions, such as tuberculosis, pulmonary infarction, radiation injury, chemotherapeutic drugs, pneumonia, interstitial lung disease, etc. The cells are present in tight papillary clusters (**Fig. 12.10**). Individual cells show a moderate amount of vacuolated cytoplasm. Nuclei are mildly enlarged with fine chromatin and single to multiple prominent nucleoli.

The hyperplastic Type II pneumocytes are the common source of false positive diagnosis as adenocarcinoma.[5]

INFECTIONS

Cytological examination of the respiratory samples play good role in the diagnosis of various infective processes.

Bacterial Infections

Large varieties of Gram-positive and Gram-negative organisms cause bacterial pneumonia. The common organisms causing bacterial pneumonia are *Staphylococcus aureus*, *pneumococci*, *Streptococcus*, *Klebsiella pneumoniae*, *Pseudomonas*, *Haemophilus influenzae*, etc. These organisms are identified by Gram stain and corresponding mycobacterial culture. The sputum or other respiratory specimen shows abundant polymorphs, necrotic debris and degenerated bronchial epithelial cells. Cytologic

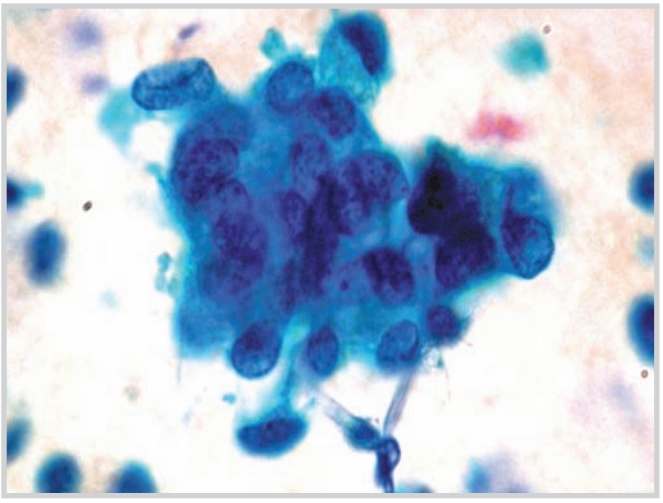

Fig. 12.10: Tight papillary cluster of cells with vacuolated cytoplasm in hyperplastic pneumocytes (Papanicolaou's stain X HP)

BOX 12.10 Mycobacterial infection

- Fine needle aspiration cytology (FNAC) of lung lesion is mostly done in suspicion of malignancy
- Cytology:
  - Multiple epithelioid cell granulomas
  - Multinucleated Langhans type giant cells
  - Necrosis

Confirmation
- Ziehl-Neelsen stain shows acid-fast bacilli
- Atypical mycobacteria may stain weakly. Fite stain is better
- Culture
- Polymerase chain reaction (PCR) confirmation

examination is usually not performed in clinically diagnosed cases of bacterial pneumonia.

Tuberculosis

Mycobacterium tubercle (**Box 12.10**) infection of the lung provokes granulomatous inflammation. The cytology smear shows multinucleated giant cells, epithelioid cell granulomas, lymphocytes and necrosis (**Figs 12.11** and **12.12**). In sputum sample, the combination of multinucleated giant cells and epithelioid cell granulomas are pathognomonic for tuberculosis and can be seen in 60% tuberculosis cases.[6] However, these features are not specific for tuberculosis and may be noted in other granulomatous inflammations, such as fungal infections, sarcoidosis, etc. Ziehl-Neelsen stain for acid-fast bacilli or culture is necessary for confirmation.

Actinomycosis

Actinomyces species are commonly present in tonsilar crypts and gingival crevices. The organisms are usually noted as a contaminant in the sputum.

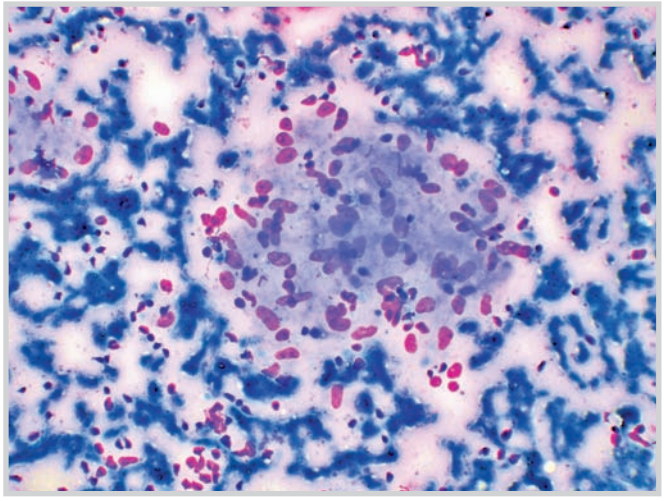

Fig. 12.11: Well-formed epithelioid cell granuloma in a case of tuberculosis of lung (May-Grunwald Giemsa stain X MP)

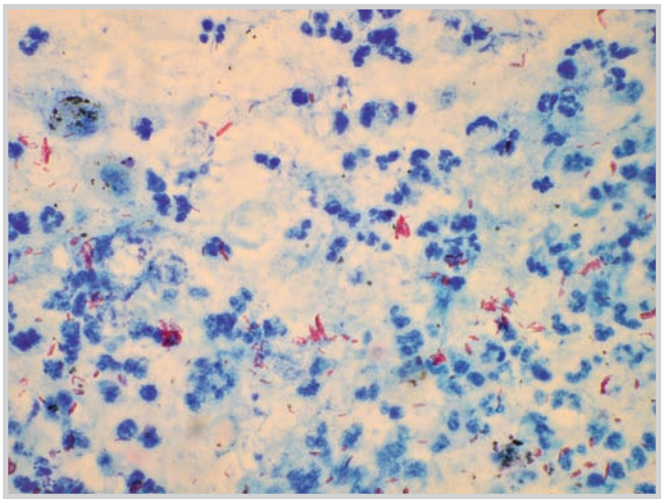

Fig. 12.12: Strong positivity of acid-fast bacilli in case of tuberculosis of lung (Ziehl-Neelsen stain X OI)

They infect lung in anaerobic condition such as lung injury or infarct. The organisms are cotton ball-like floppy hemtoxylin-stained tangled filamentous material with associated squamous cells. Background of the smear often shows polymorphonuclear leukocyte reaction. The presence of actinomycosis organisms in the BAL or FNAC material usually indicates infection.

Nocardia

Nocardia is the aerobic filamentous organisms and is commonly the inhabitant of soil. They infect lung in the immunocompromised patient. *Nocardia* is slender, filamentous, branching organisms. They are Gram-positive and weak acid-fast positive organism. They are usually diagnosed by BAL specimen.

Fungal Infections

Fungal infections (**Box 12.11**) are readily diagnosed in respiratory specimen because of the typical morphology of the fungi and most of the fungi are detectable in May-Grünwald (MGG) stain; however, special stains and fungal culture may be needed for the final confirmation. On cytology smear, different substances can be misinterpreted as fungus, such as pollen grains, anthracotic pigment, talc and red blood cells (**Table 12.4**).

The smears usually show multinucleated giant cells, epithelioid cell granulomas, polymorphs and eosinophils along with necrotic material. Morphological characteristics of different fungi are mentioned in **Box 12.11**.

Aspergillus

Aspergillus infection of the lung may cause extrinsic allergic alveolitis, allergic bronchopulmonary aspergillosis, infection

TABLE 12.4: Substances that simulate fungus

Materials	*Comments*
RBCs: Variable depigmentation during Papanicolaou stain and may simulate fungal spore	RBCs are present as clusters
Nuclear lobes of polymorphs	• They are of variable size • Smaller sized • Block-like nuclear chromatin
Talc	• Central hole or defect • Present in polarized light
Hemosiderin	• Variable-sized particle • Absence of internal structure of fungus

BOX 12.11 Common fungal infections

- *Aspergillus*: Septate, uniform acute angle branching, 30 micron width hyphae, septate

Special stain: PAS, mucicarmine, methanamine silver

- Zygomycetes: Broad, nonseptate, wide-angle branching

Special stain: PAS, mucicarmine

- *Cryptococcus*: 5–10 µ diameter round, thick outer capsule, narrow-based budding

Special stain: India ink,

- *Histoplasma*: Small round (2–5 µ), narrow budding, usually inside the macrophages

Special stain: PAS, Z-N stain

- *Candida*: Thin slender pesudohyphae with budding yeast

Special stain: PAS

Abbreviations: PAS—periodic acid-Schiff; Z-N—Ziehl–Neelsen

in the already existing lung cavity, tracheobronchitis and diffuse parenchymal infection of the lung or solitary cavitary lesion. The *Aspergillus* infection usually occurs in severely immunocompromised patients particularly AIDS patient. The organism is usually invasive and enters the blood stream by invasion into the blood vessel. The organism is long septate, uniform and acute angle branching. Intracavitary aspergillosis may show ball-like aggregation of *Aspergillus*, calcium oxalate crystal and squamous cell atypia. The atypical squamous cells may simulate malignant cell because of nuclear enlargement, pleomorphism and hyperchromasia.

Pulmonary Zygomycosis

These include *Mucor*, *Absidia*, *Cunninghamella*, and *Rhizopus*. The infection usually occurs in debilitated patients, diabetes mellitus, renal failure and immunocompromised patient. Zygomycosis group of fungi overall show broad, nonseptate, wide angle branching hyphae (**Fig. 12.13**).

Pulmonary Cryptococcosis

Pulmonary cryptococcosis is caused by *Cryptococcus neoformans*. *Cryptococcus neoformans* is found in soil contaminated by bird droppings. It causes systemic infection, and lung is also involved. The organism is 5–10 μ diameter round with thick outer capsule (**Fig. 12.14**). Narrow-based budding is often noted. Special stains, such as periodic acid-Schiff, mucicarmine or alcian blue are needed for better visualization of the fungi.

Histoplasma

Histoplasma is the inhabitant of soil. Histoplasmosis is caused by inhalation of spore of *Histoplasma capsulatum*. Infection occurs particularly in immunocompromised patient and debilitated person. The organism is intracellular and remains within the macrophages. The organism is small round 2–5 microns in diameter and therefore difficult to recognize in Papnicolaou's stained smear; however, they are well visualized in MGG staining (**Fig. 12.15**). Special stains such as methanamine silver is needed to demonstrate the organism.

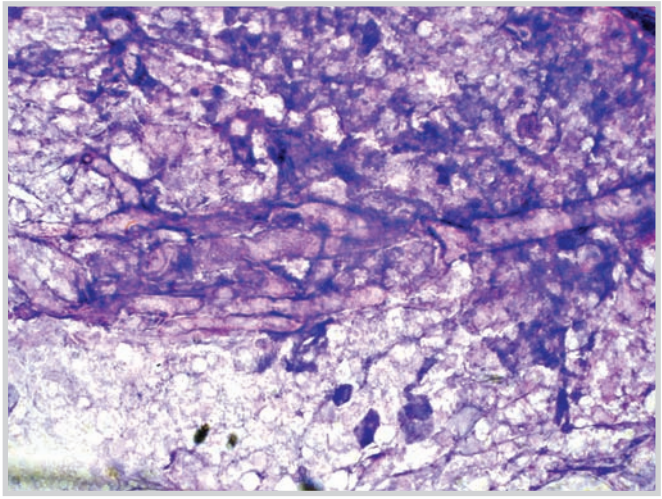

Fig. 12.13: Broad, non-septate, wide angle branching hyphae of mucormycosis of lung infection (May-Grunwald Giemsa stain X HP)

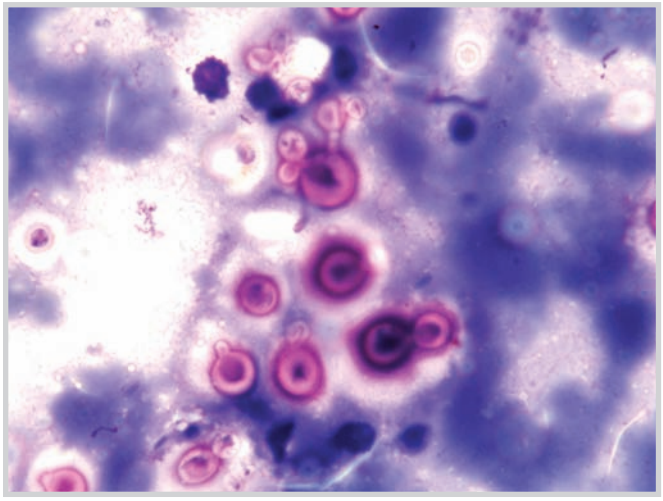

Fig. 12.14: Round capsulated *Cryptococcus* in lung infection (May-Grunwald Giemsa stain X OI)

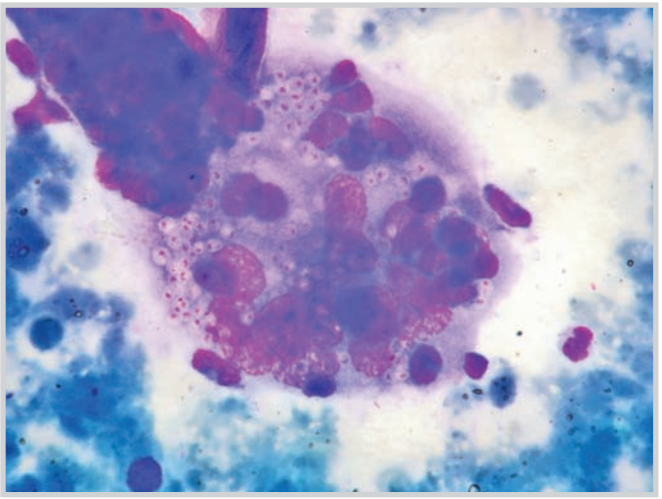

Fig. 12.15: Small round intracellular *histoplasma* within the histiocyte (May-Grunwald Giemsa stain X OI)

Pulmonary Candidiasis

Candidiasis is caused mainly by *Candida albicans*. Candidiasis is an opportunistic infection and occurs in immunocompromised patient. *Candida* appears as small, oval, 2 to 4 μm budding yeasts or elongated pseudohyphae forms (**Fig. 12.16**). In the healthy person, *Candida* is the commensal organism of the oral cavity and upper respiratory tract. Therefore, the simple presence of candidal spore may not indicate infection. However, the presence of candidal hyphae in the FNAC of lung indicates candidiasis.

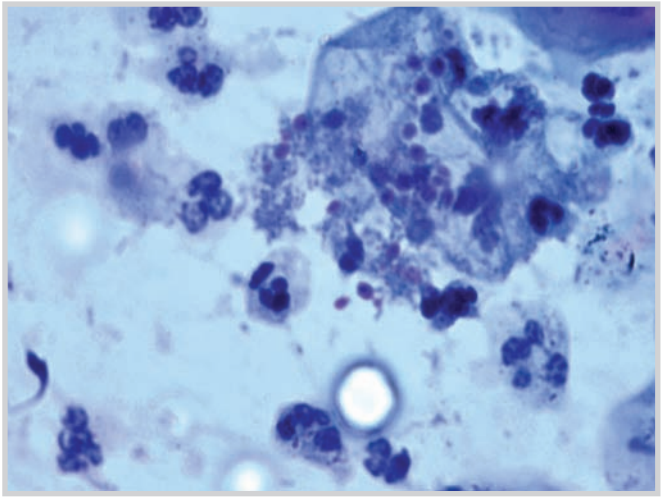

Fig. 12.16: Candidal spores and inflammatory cells in brochioalveolar lavage sample (Papanicolaou's stain X OI)

BOX 12.12 ***Pneumocystis carinii* infection**

- Associated with immunocompromised patient, particularly in AIDS
- Best sampling method: bronchioloalveolar lavage
- Tissue response
  - Alveoli filled with frothy eosinophilic material
  - Granulomatous inflammation
  - Giant cell reaction
  - No tissue response
- Cytology
  - Frothy appearance
  - Extracellular aggregate
  - Not stained by Papanicolaou's staining
  - Cup-shaped structure of 6–8 µm diameter
  - One surface flat and a central dark zone
- Special stain
  - Methanamine silver
- Mimickers
  - Lubricant
  - RBCs in aggregates
  - Mucus

Pneumocystis carinii Infection

Pneumocystis carinii infection (**Box 12.12**) is seen in immunocompromised patient particularly in AIDS. Bronchoalveolar lavage is the best way to diagnose *Pneumocystis carinii* infection. The cytology smear may show granulomatous inflammation and giant cell reaction. The organisms are not visualized in the PAP-stained smear. However, masses of organisms are embedded in eosinophilic amorphous foamy proteinaceous alveolar cast. In a proper clinical setting, this finding should be taken as suspicious. Special stains like methanamine silver should be done to demonstrate the organism. *Pneumocystis carinii* is a cup-shaped structure of 6–8 µm diameter with one surface flat and a central dark zone. With the help of MGG stain, intracystic trophozoites may be seen within the cyst.[7,8]

Viral Infection

Herpes Virus Infection

Herpes simplex virus (HSV) is a DNA virus and causes tracheobronchitis, pharyngitis and lung infection in immunocompromised, diabetic and debilitated patient. The cytological examination of sputum in HSV infection shows many multinucleated giant cells. The individual cells show multiple enlarged basophilic nuclei with ground-glass appearance, slaty gray homogenized chromatin and large intranuclear eosinophilic inclusions. Nuclei of the giant cells are molded.

Cytomegalovirus

It is a DNA virus and affects immunocompromised and debilitated patient. The patient presents with fever, dyspnea and cough. Cytology smears show markedly enlarged cells with enlarged nuclei. Large amphophilic intranuclear inclusion with a peripheral clear halo and margination of chromatin on the inner nuclear membrane are seen in the bronchial cells, macrophages and endothelial cells. Small satellite basophilic cytoplasmic inclusions are seen. Occasional multinucleated cells are also noted along with the mononuclear cells.

Adenovirus

Adenovirus usually causes mild fever. The cytology smears show cilliocytophoria of the respiratory epithelial cells. The virus affected cells show two types of inclusions. The first type shows small reddish well-circumscribed inclusion surrounded by clear halo and other type shows large basophilic homogenous inclusion that completely fills the entire nucleus.

Measles Virus

This is an RNA virus and commonly infects children. It is a self-limited infection and rarely becomes fatal except in immunocompromised patient. Cytology smear shows enormous multinucleated giant cells (Warthin-Finkelday cells) with more than 100 nuclei containing eosinophilic intracytoplasmic and intranuclear inclusions.

Parasitic Infection

Strongyloidiasis

Strongyloid stercoralis infection is caused by penetration of larva through the skin. The larva penetrates the blood vascular wall, comes to the circulation and resides in the intestine. It comes to the lung through hematogenous migration from the intestine or skin. The larvae are long, 400 microns in length with a characteristic V-shaped notch in the tail.

LUNG CARCINOMAS[5-11]

Carcinoma of the lung is the most frequent cancer in the world. It is the commonest cause of death in males due to cancer.[9] Male to female ratio of lung cancer is 2.7:1. The peak age of lung cancer is 60–70 years. Lung cancer usually occurs after 40 years of age. Large amount of evidences suggest that the major etiological factor of lung cancer is cigarette smoking.[10,11] The smoke of cigarette and other tobacco products contain a large number of carcinogens and toxic products. Inhalation of smoke causes inflammation of the respiratory epithelium. Cigarette smoking increases the risk of all lung cancer, but it is particularly associated with squamous cell carcinoma followed by small cell carcinoma and adenocarcinoma.

The common complaints of the patient are progressive shortness of breath, chest pain, cough, dyspnea and hemoptysis. Infections of lung due to bacterial, viral or fungal agents may also produce space occupying lesions and may yield hyperplastic atypical bronchial cells. Reactive atypical cells are usually in cohesive clusters, scanty in number and have bland nuclear chromatin. The cells often preserve cilia. **Table 12.5** highlights the differentiating points between reactive atypical bronchial cells from malignant cells.

Classification of Lung Cancer

World health organization (WHO) classified lung carcinoma[12] as mentioned in **Box 12.13**. The box shows a mild modification of this classification.

Squamous Cell Carcinoma

This is one of the commonest lung cancers. It is strongly associated with cigarette smoking, and more than 90% of squamous cell carcinomas (SQC) of lungs occur in cigarette smokers. The majority of SQC arises centrally from major bronchi or segmental bronchi and only 10% occur in the periphery. The salient cytological features are highlighted in **Box 12.14**.

Cytology: Cytological examination of sputum provides a quick diagnosis of SQC. Cytology smear shows clusters and discrete malignant cells in the background of necrosis and granular debris. The individual malignant cells are oval to polyhedral with centrally placed nuclei (**Figs 12.17** to **12.19**). The cytoplasm is moderate to abundant and shows orange color in PAP stain. The dense orangeophilic cytoplasm of the cell is particularly noted in keratinizing SQC (**Table 12.6**). Keratinized cells are more evident in sputum than in bronchial washing or brushing specimen.

Nuclei show moderate to marked pleomorphism and marked hyperchromasia. Nuclear chromatin is irregular and coarse. Pyknotic dense ink dot-like nuclear chromatin is more frequent in sputum samples. Nucleoli are usually not visible. In addition, many tadpole cells and fiber-like cells may be noted (**Fig. 12.20**).

TABLE 12.5: Differentiating points between reactive atypical bronchial cells from malignant cells

Features	*Reactive atypical bronchial cells*	*Malignant cells*
Cell arrangement	Predominantly tight papillary clusters	Dissociated cells
Margin of the cluster	Smooth	Irregular
Nuclear crowding	No significant nuclear overlapping	Nuclear overlapping
Atypical cells	Scanty	Many
Cilia	Usually present	Ciliocytophoria
Nuclear margin	Regular	Irregular
Nuclear chromatin	Fine	Irregular coarsely clumped

BOX 12.13 WHO classification of lung tumors

- Squamous cell carcinoma
  - Papillary
  - Clear
  - Small cell
  - Basaloid
- Small cell carcinoma
  - Combined small cell carcinoma
- Adenocarcinoma
  - Acinar
  - Papillary
  - Bronchioloalveolar
  - Solid adenocarcinoma
- Large cell carcinoma
  - Large cell neuroendocrine carcinoma
  - Combined large cell neuroendocrine carcinoma
  - Basaloid carcinoma
  - Lymphoepithelioma-like carcinoma
  - Clear cell carcinoma
  - Large cell carcinoma with rhabdoid phenotype
- Adenosquamous carcinoma
- Sarcomatoid carcinoma
- Carcinoid
  - Typical carcinoid
  - Atypical carcinoid
- Salivary gland carcinoma

BOX 12.14 Squamous cell carcinoma

- Polyhedral cells
- Eosinophilic cytoplasm
- Intracellular keratin (orangeophilic cell)
- Round nucleus with moderate nuclear pleomorphism
- Hyperchromatic nucleus, inconspicuous nucleoli
- Fiber cells and tadpole cells
- Ghost of squamous cells
- Background necrosis and granular debris
- Keratin pearls

Immunocytochemistry: Positive for CK 5/6, CEA. Usually negative for TTF

Abbreviations: CK—cytokeratin; CEA—carcinoembryonic antigen; TTF—thyroid transcription factor

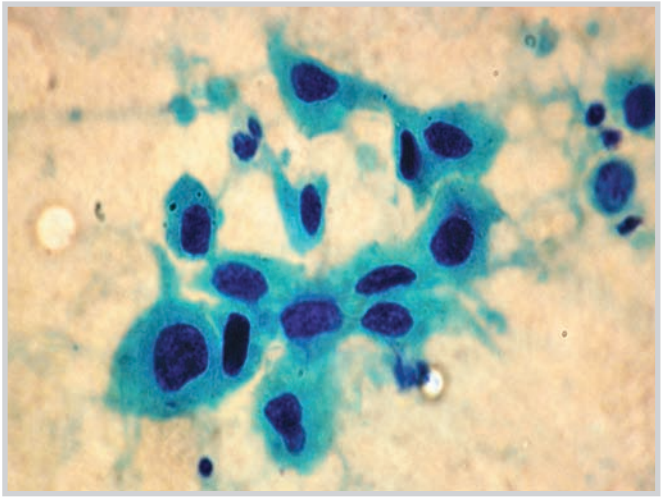

Fig. 12.17: Oval to polyhedral cells with moderately pleomorphic nuclei in squamous cell carcinoma of lung (Papanicolaou's stain X HP)

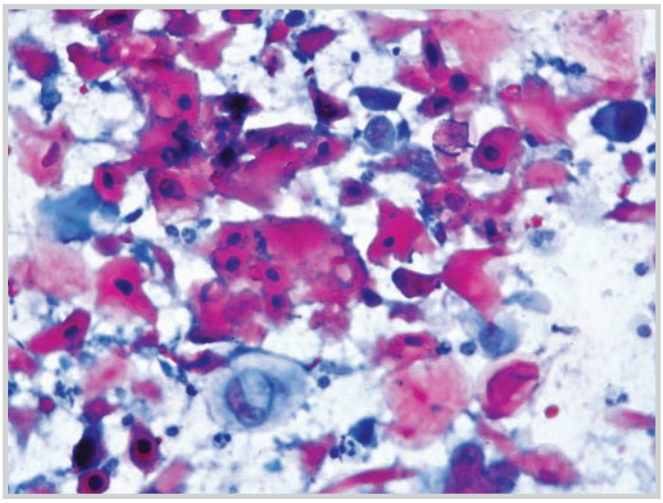

Fig. 12.19: Polyhedral cells with orangeophilic cytoplasm and dark hyperchromatic nuclei in squamous cell carcinoma of lung (Papanicolaou's stain X HP)

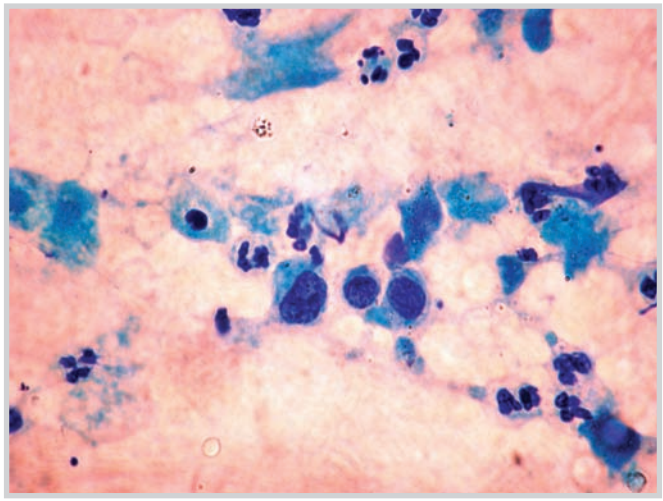

Fig. 12.18: Polyhedral cells with large hyperchromatic nuclei in squamous cell carcinoma of lung (Papanicolaou's stain X HP)

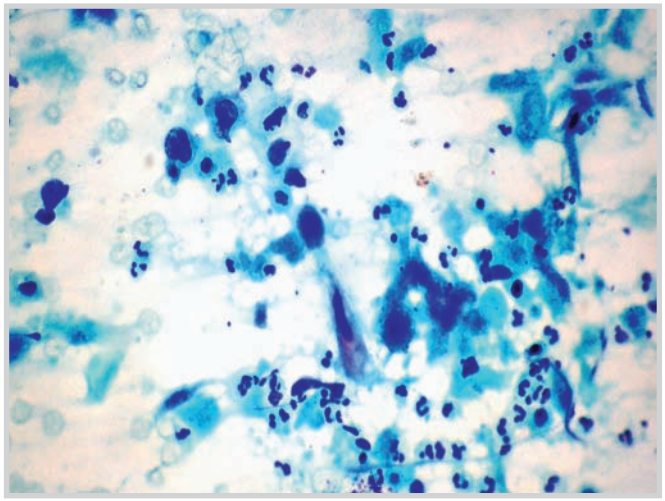

Fig. 12.20: Fiber-like cell with elongated nuclei in in squamous cell carcinoma of lung (Papanicolaou's stain X HP)

TABLE 12.6: Differentiating features between keratinizing squamous cell carcinoma and nonkeratinizing squamous cell carcinoma

Cytology features	*Keratinizing squamous cell carcinoma*	*Nonkeratinizing squamous cell carcinoma*
Cell clusters	Less, more discrete cells	More clusters
Cytoplasm	Orangeophilic	Basophilic
N/C ratio	Low	High
Nucleoli	Absent	Prominent
Chromatin	Coarse	Fine
Pyknotic nuclei	Frequent	Absent
Fiber and tadpole cells	More frequent	Less frequent

BOX 12.15 Differential diagnosis of squamous cell carcinoma

- Squamous metaplasia: Monomorphic small round nuclei with homogenous chromatin
- Reactive squamous atypia: Monomorphic with regular nuclear margin
- Vegetable bodies: Regular cell with thick cellulose wall
- Small cell carcinoma: Hyperchromatic nuclei, absence of nucleoli, crushing artifact, molding
- Adenocarcinoma: Cytoplasmic vacuoles, fine nuclear chromatin, prominent nucleoli
- Metastatic squamous cell carcinoma: Clinical history

Occasionally, keratin pearls are seen. The ghost of squamous cells along with necrosis in a clinically suspicious patient may suggest the possibility of malignancy.

In moderately and poorly differentiated SQCs, the individual cells may not show features of keratinization and the cytoplasm is less eosinophilic and scanty in amount. The nuclear size is relatively large with more open chromatin and prominent nucleoli. Pyknotic nuclei are less frequent in nonkeratinizing SQC.

Diagnostic difficulties: Cytological features of SQC are characteristic; however, at times different conditions mimic this entity (**Box 12.15**).

- Squamous metaplasia: Squamous metaplasia occurs due to chronic bronchial irritation. These cells are smaller in size with relatively high N/C ratio. Metaplastic squamous cells may simulate SQC; however, these cells have monomorphic small round nuclei and smooth nuclear margin with homogenous chromatin.
- Reactive squamous atypia: Reactive squamous atypia are noted in cavitary *Aspergillus* lesions, radiation therapy, chemotherapeutic drugs and infection of the lung. The nuclei of these atypical cells are monomorphic with regular nuclear margin.
- Vegetable bodies: Thick-walled plant cells with dark nuclei may rarely be mistaken as SQC of the lung. However, the plant cells are very regular and their cellulose wall is thick.
- Small cell carcinoma: Small cell nonkeratinizing SQC may simulate small cell carcinoma because of relatively smaller size and lack of typical squamous differentiation. Coarse chromatin, prominent nucleoli, absence of crushing artifact and nuclear molding are the characteristic differentiating points between small cell carcinoma and SQC.
- Adenocarcinoma: At times, poorly-differentiated adenocarcinoma is difficult to differentiate from poorly differentiated SQC. Adenocarcinoma cells often show cytoplasmic vacuoles that are positive for mucin. Nuclei of the cells show fine chromatin and prominent nucleoli.
- Metastatic squamous cell carcinoma: Without a proper history of another SQC, it is almost impossible to differentiate a primary from a metastatic SQC on cytological basis.

Adenocarcinoma

Adenocarcinomas of the lung are the most common subtype in females. These are commonly located in the peripheral part of the lung and may be detected in an asymptomatic patient. WHO has defined four major subtypes of adenocarcinoma: (1) acinar, (2) papillary, (3) bronchioloalveolar and (4) solid with mucus formation. Among all these subtypes of adenocarcinoma, bronchioloalveolar needs special attention.

BOX 12.16 Adenocarcinoma of lung

- Discrete, cluster and glandular pattern
- Sheets of cells with honeycomb pattern
- Round cells with moderate vacuolated cytoplasm
- Central to eccentric nucleus
- Fine chromatin
- Prominent nucleoli

The salient cytological features of adenocarcinoma are mentioned in **Box 12.16**.

Cytology: The cytology smears of adenocarcinoma show single and small clusters of cells (**Figs 12.21** to **12.24**). The cells are occasionally arranged as acini or honeycomb-like sheets. The gland may not be well formed and is often absent. There may be three-dimensional cell balls of malignant cells with nuclear overcrowding. The cytoplasm of the cell is moderate to abundant and often contains large vacuoles filled with mucin. Large vacuoles may push the nucleus to the periphery of the cell causing indented margin of the nucleus. The background mucinous material may also be noted in a small number of cases (less than 25%). The nuclei are central to eccentric in position with smooth margin having moderate pleomorphism. Nuclear chromatin is fine and evenly distributed. In poorly-differentiated adenocarcinoma, nuclear chromatin is coarse and hyperchromatic. Nucleoli are usually prominent and characteristically large and regular. One important feature of adenocarcinoma is the presence of nucleocytoplasmic polarity. The nuclei of the cells are almost always in basal position.

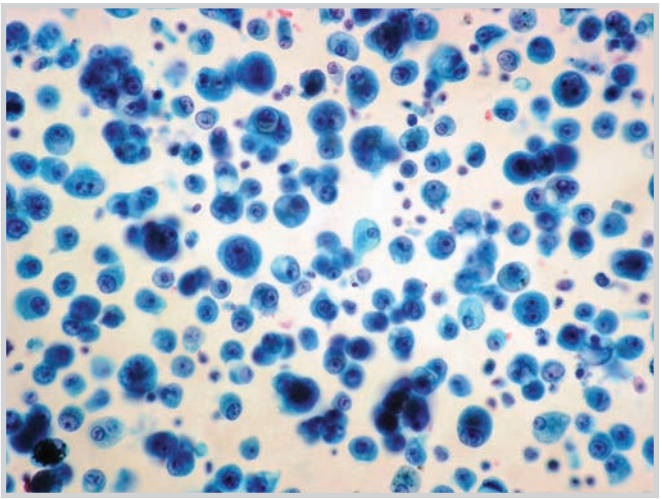

Fig. 12.21: Brochioalveolar lavage sample shows discrete and loose clusters of malignant cells in adenocarcinoma of lung (Papanicolaou's stain X MP)

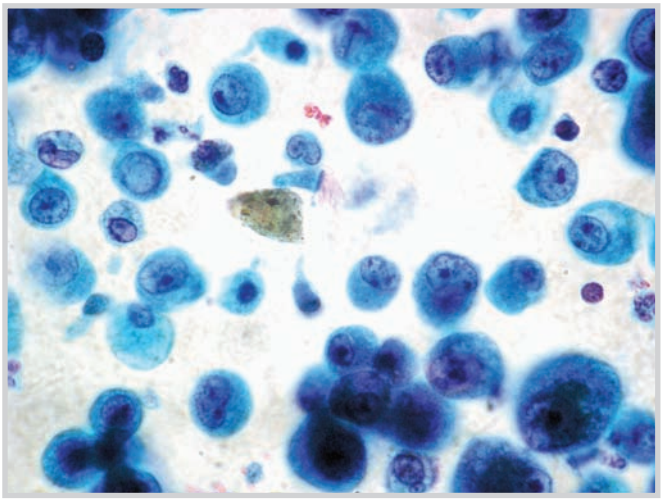

Fig. 12.22: Brochioalveolar lavage sample shows malignant cells with moderate amount of cytoplasm and enlarged nuclei with prominent nucleoli in adenocarcinoma of lung (Papanicolaou's stain X HP)

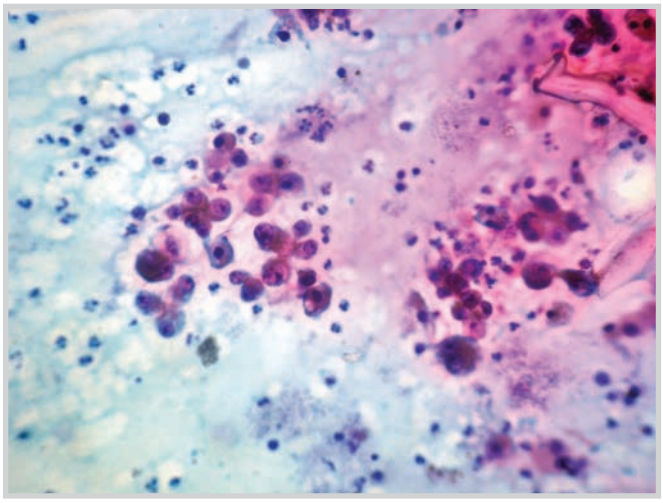

Fig. 12.23: Discrete malignant cells entangled in mucous in adenocarcinoma of lung (Papanicolaou's stain X MP)

Bronchioloalveolar Carcinoma

Bronchioloalveolar carcinoma (BAC) typically occurs in the peripheral part of the lung as a single nodule, multiple nodules or diffuse lesions. The key cytological features are mentioned in **Box 12.17**.

Cytology

Bronchioloalveolar carcinoma may be mucinous and nonmucinous types with overlapping cytomorphology. The cells are often arranged in monolayered sheets, ball-like tight clusters and papillae without any fibrovascular core. Cells of the mucinous type of BAC show abundant vacuolated cytoplasm with monomorphic nuclei having prominent nucleoli. In comparison, the cells of nonmucinous type of BAC show smaller cells with relatively pleomorphic nuclei (**Figs 12.25** to **12.27**). Intranuclear

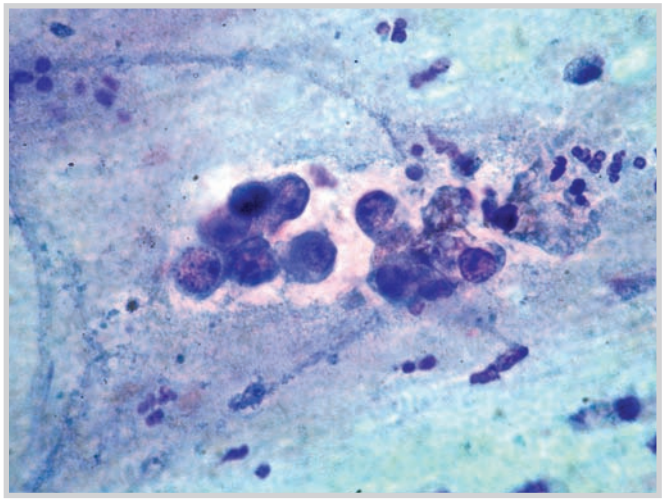

Fig. 12.24: High-powered view shows round cells with moderate amount of cytoplasm and prominent nucleoli (Papanicolaou's stain X HP)

BOX 12.17 Bronchioloalveolar carcinoma

- Monolayer sheet, ball-like clusters
- Papillary fragments of cells
- Round to oval cells with mild to moderate vacuolated cytoplasm
- Mild to moderate nuclear atypia
- Finely granular nuclear chromatin
- Inconspicuous nucleoli
- Intranuclear inclusions

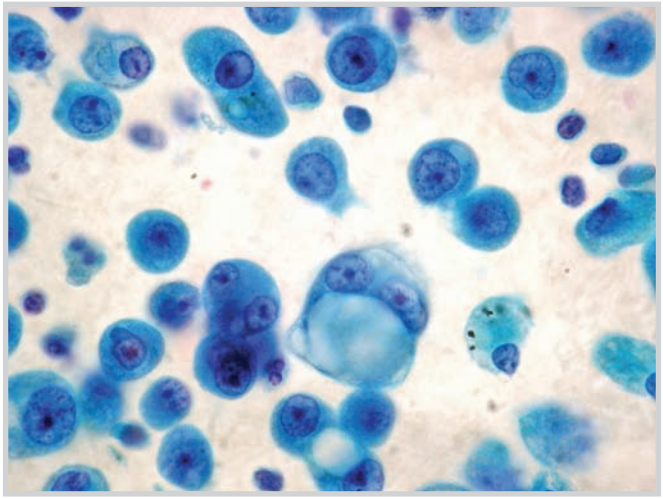

Fig. 12.25: Brochioalveolar lavage sample shows small clusters and discrete cells with abundant vacuolated cytoplasm and enlarged nuclei with prominent nucleoli in a case of bronchioloalveolar carcinoma (Papanicolaou's stain X HP)

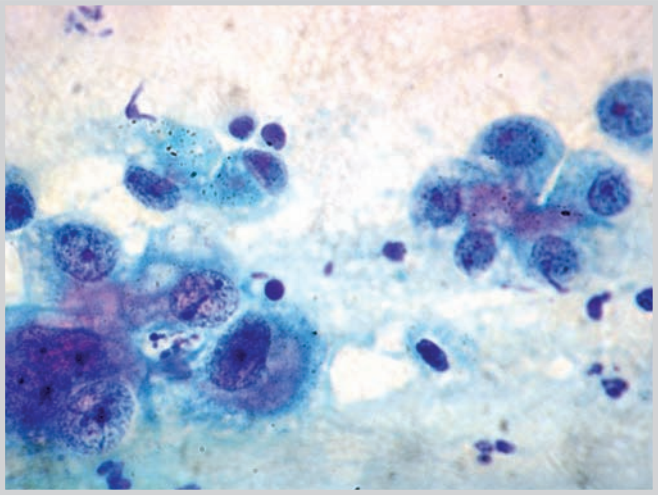

Fig. 12.26: Smear shows malignant cells with abundant vacuolated cytoplasm in a case of bronchioloalveolar carcinoma. Nuclei are mildly pleomorphic with single to multiple prominent nucleoli (Papanicolaou's stain X HP)

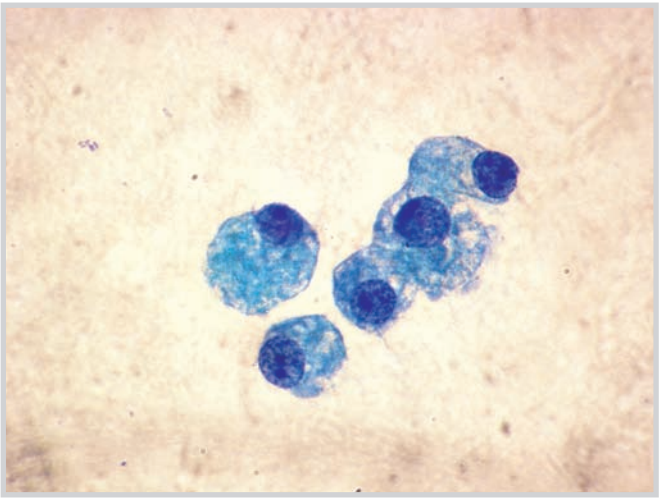

Fig. 12.27: Discrete cells with minimal alteration of nucleocytoplasmic ratio in bronchioloalveolar carcinoma (Papanicolaou's stain X HP)

cytoplasmic inclusions are one of the diagnostic features of BAC. Psammoma bodies are present in occasional cases.

Differential Diagnosis

Various differential diagnosis of adenocarcinoma is highlighted in the **Box 12.18**.

- Poorly-differentiated squamous cell carcinoma: At times, poorly-differentiated adenocarcinoma is difficult to distinguish from non-keratinizing SQC. The problem has been discussed in the previous section.
- Metastatic adenocarcinoma: It is not possible to differentiate primary from a metastatic adenocarcinoma on the basis of cytology alone. A clinical history may be helpful in this aspect.
- Reactive bronchial cell hyperplasia: This has been highlighted previously in the **Table 12.5**. The clusters of reactive atypical cells have a more regular outline. There is no significant nuclear overlapping. In addition, the individual cells show cilia. The nuclei of the cells are also monomorphic with regular margin.
- Goblet cell hyperplasia: Goblet cells with large vacuolated cytoplasm may mimic adenocarcinoma. However, these cells have monomorphic benign looking nuclei that help in diagnosis.
- Reactive mesothelial cells: The mesothelial cells often show nuclear enlargement and pleomorphism with prominent nucleoli. Mesothelial cells are usually arranged in monolayerd sheets unlike three-dimensional clusters in adenocarcinoma. The individual cells show a typical window-like pattern. Differential diagnosis of adenocarcinoma and reactive mesothelial cells has been discussed in detail in the next section.
- Vegetable cells: Vegetable cells are recognized by their thick cell wall.
- Hyperplasia of pneumocytes: At times, there may be hyperplasia of pneumocytes Type II that may resemble BAC.
- Hamartoma: This may be a source of false positive diagnosis as hamartoma contains good amount of glandular material. The presence of myxofibroid tissue, cartilaginous fragments and cells with preserved cilia helps in the diagnosis of hamartoma.
- Viral inclusions: At times, large viral inclusions may mimic prominent nucleoli and may be mistaken as adenocarcinoma. However, other features such as halo around inclusion and beaded chromatin are helpful differentiating features.

BOX 12.18 Differential diagnosis of adenocarcinoma and bronciolalveolar carcinoma

- Poorly differentiated squamous cell carcinoma: Cytoplasmic vacuoles, fine nuclear chromatin, prominent nucleoli
- Metastatic adenocarcinoma: Impossible without clinical history
- Reactive bronchial cell hyperplasia: Clusters have regular outline, Individual cells show presence of cilia. Nuclei are monomorphic with regular margin
- Goblet cell hyperplasia: Monomorphic nuclei
- Reactive mesothelial cells: Honeycomb sheet, window pattern and regular nuclear margin
- Vegetable cell: Thick cell wall
- Hamartoma: Presence of myxofibroid tissue, cartilaginous fragments and cells with preserved cilia helps in the diagnosis of hamartoma
- Viral inclusions: Halo around inclusion and beaded chromatin are helpful differentiating features

Small Cell Carcinoma

Small cell carcinoma accounts for 20% of lung cancer and 90% of these tumors are centrally located. It is strongly associated with cigarette smoking. Male to female ratio of this tumor is 2:1. The WHO has classified small cell carcinoma as (1) pure small cell carcinoma and (2) combined small cell carcinoma with other carcinomas. This is a highly aggressive malignancy and metastasizes quickly. The surgical resection is ineffective in this malignancy. This tumor is sensitive to chemotherapy. The key cytological features are mentioned in **Box 12.19**.

BOX 12.19 Small cell carcinoma

- Dissociated cells
- Small cells resembling lymphocytes
- Scanty cytoplasm
- Hyperchromatic nucleus with inconspicuous nucleoli
- Nuclear molding
- Paranuclear blue bodies
- Crushing artifact and nuclear threading

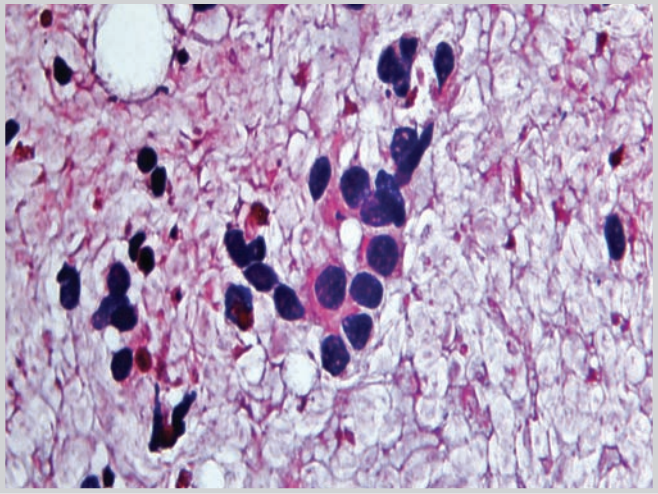

Fig. 12.28: Small row of malignant cells in small cell carcinoma of lung (Papanicolaou's stain X HP)

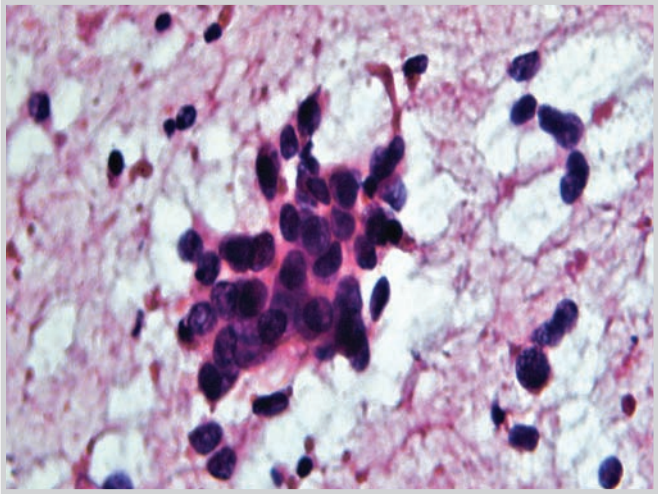

Fig. 12.29: Malignant cells with scanty cytoplasm and hyperchromatic nuclei in small cell carcinoma of lung (Papanicolaou's stain X HP)

Cytology

The cytology smears of the small cell carcinoma shows a predominant discrete population of the small round cells about one and half times larger than mature lymphocytes. In sputum smears, the cells are entangled in the mucus and arranged in small rows like Indian file arrangement (**Figs 12.28** to **12.30**). The tumor cells show nuclear threading and crushing artifact due to the fragile nature of the cells. The individual cells are round with scanty cytoplasm. Occasional spindle cells are also seen. The nuclei are relatively monomorphic with finely granular "salt and pepper" like chromatin. Nucleoli are absent. Nuclear molding is characteristic of small cell carcinoma. Cytoplasm may contain round light blue to dark homogenous bodies known as paranuclear blue bodies. In the sputum sample, the detailed morphology of the cells may not be seen as they are enmeshed in mucus. However, presence of small round cells with hyperchromatic nuclei and scanty cytoplasm indicate the diagnosis of small cell carcinoma. In comparison, bronchial washing or brushing specimen show better nuclear morphology. The nuclei show cell to cell compression and characteristic nuclear molding.

Immunocytochemistry

The small cell carcinoma is positive for CD56, chromogranin, synaptophysin, cytokeratin and thyroid transcription factor-1 (TTF-1).

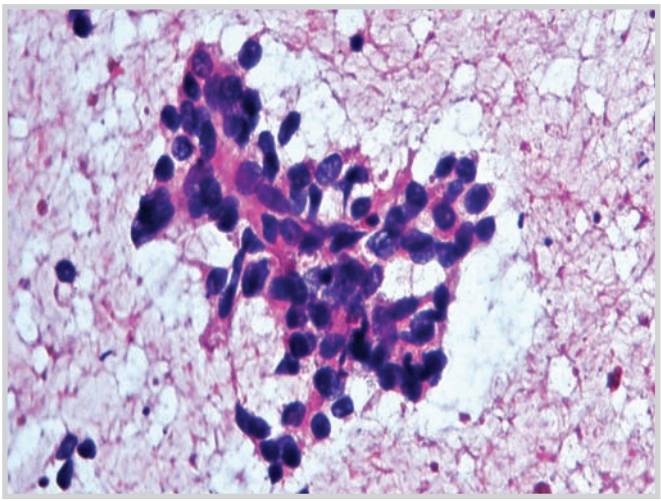

Fig. 12.30: Malignant cells are often compressed with each other in small cell carcinoma of lung (Papanicolaou's stain X HP)

Differential Diagnosis

- Reserve cell hyperplasia: Reserve cells show much smaller cells with smudged nuclei and featureless chromatin.
- Lymphocytes: Lymphocytes in chronic inflammation may be mistaken as small cell carcinoma. Lymphocytes are

usually dissociated and smaller than the cells of small cell carcinoma.

- Poorly-differentiated carcinoma: Poorly differentiated SQC and adenocarcinoma with small round cells may be confused with small cell carcinoma. Cellular dissociation, scanty cytoplasm and nuclear molding favor the diagnosis of small cell carcinoma.
- Carcinoid tumor: Discrete cells with relatively monomorphic nuclei may pose a diagnostic dilemma with carcinoid tumor. However, the presence of abundant granular cytoplasm, salt and pepper chromatin, and prominent nucleoli favor the diagnosis of carcinoid.
- Lymphomas: Small round dissociated cells with scanty cytoplasm may simulate non-Hodgkin's lymphoma (NHL). The presence of lymphoglandular bodies is characteristic of NHL.
- Nonkeratinizing SQC: Small cells of nonkeratinizing SQC may be difficult to distinguish from small cell carcinoma. However, nuclear morphology is helpful in the diagnosis of small cell carcinoma.

Table 12.7 shows differential diagnosis of small cell carcinoma of the lung.

Undifferentiated Large Cell Carcinoma

Large cell carcinoma (LCC) accounts for 10% of all lung carcinomas. These are undifferentiated LCCs without any particular evidence of differentiation toward squamous or adenocarcinoma. Majority of LCCs are located in the peripheral part of lung. WHO classified LCC as (1) large cell neuroendocrine carcinoma, (2) combined large cell neuroendocrine carcinoma, (3) basaloid carcinoma, (4) lymphoepithelioma-like carcinoma, (5) clear cell carcinoma, and (6) LCC with rhabdoid phenotype. Overall the key cytological features of LCC are mentioned in **Box 12.20**.

Cytology

The cells are usually arranged in syncytial clusters and also discretely. No acinar or glandular differentiation is seen. The individual cells show moderate to abundant dense cytoplasm with ill-defined margins. Nuclei show marked pleomorphism, nuclear margin irregularity, irregular coarse chromatin and multiple enlarged prominent nucleoli. Many large mono/multinuclear

BOX 12.20 Undifferentiated large cell carcinoma

- Large cell with marked nuclear pleomorphism
- Moderate to abundant cytoplasm
- Ill-defined cytoplasm
- Severely pleomorphic bizarre nuclei
- Multiple prominent nucleoli
- Large tumor giant cell
- Polymorphs sticking to the cells

TABLE 12.7: Differential diagnosis of small cell carcinoma of lung

	Small cell carcinoma	*Squamous cell carcinoma*	*Carcinoid*	*Lymphoma*
Cell clusters	Discrete	Present	Discrete	Discrete
Indian file arrangement	Present	Absent	Absent	Absent
Nuclear threading	Present	Absent	Absent	Absent
Lymphoglandular bodies	Absent	Absent	Absent	Present
Cell cytoplasm amount	Scanty to nil	Moderate	Moderate, reddish granular	Scanty
Cytoplasm character	Thin rim of basophilic	Orangeophilic dense cytoplasm	Reddish granular	Deep blue in Giemsa stain
Nuclear molding	Present	Absent	Absent	Absent
Nucleus	Small, one to two times larger than lymphocyte	Relatively low N/C ratio, small nucleus	Larger	Twice the size of mature lymphocyte
Chromatin	Granular and Salt and pepper	Irregular coarse	Salt and pepper	Fine
Immunochemistry Chromogranin Synaptophysin Thyroid transcription factor (TTF) Leukocyte common antigen (LCA)	 Positive Positive Positive Negative	 Negative Positive Mostly negative Negative	 Positive Positive Variable positivity Negative	 Negative Negative Negative Positive
Electron microscopy	Neuroendocrine granules	Desmosomal junction	Neuroendocrine granules	Nonspecific

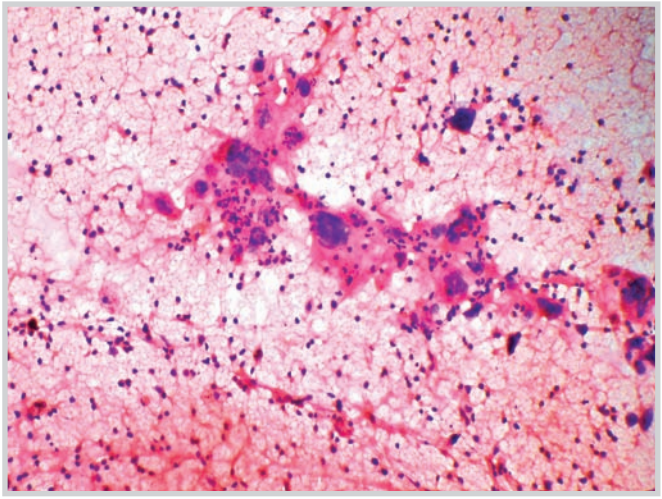

Fig. 12.31A: Discrete malignant cells with enlarged markedly pleomorphic nuclei along with many polymorphs (Papanicolaou's stain X MP)

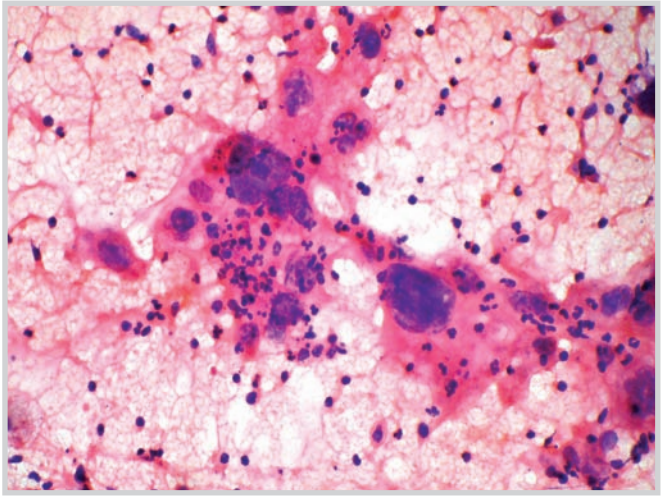

Fig. 12.31B: High-powered view of the same (Papanicolaou's stain X MP)

giant cells are also noted. The characteristic feature of LCC is sticking of polymorphs with the malignant cells (**Figs 12.31A** and **B**). The malignant cells also engulf the polymorphs. Giant cell carcinoma is one of the varieties of the undifferentiated LCC. Here, the smear shows many large multinuclear and mononuclear tumor giant cells along with heavy polymorphonuclear leucocytes infiltration. Basaloid variety of LCC shows cohesive clusters of cells. Nuclear palisading may be seen at the periphery of such aggregates of cells. Lymphoepithelioma variety of LCC shows syncytial aggregates of oval to spindle cells with large prominent nucleoli. The cells are intimately admixed with lymphocytes. The large cell neuroendocrine tumor shows a rosette-like structure and nuclear molding.

Differential Diagnosis (Box 12.21)

- Sarcomas: Large bizarre cells may give rise to the possibilities of sarcomas. However, sarcoma of the lung is rare. Immunostaining such as vimentin is helpful for confirmation of sarcoma.
- Amelanotic melanoma: Large cells with large prominent nucleoli may simulate amelanotic melanomas. Malignant melanoma cells are positive for HMB45.
- Reactive changes: Different types of lung injury may produce large bizarre reactive bronchial cells and pneumocytes. Nuclear margin irregularity and chromatin pattern help in diagnosis of LCC.
- Squamous cell carcinoma: Poorly-differentiated nonkeratinizing SQC may often show moderate nuclear enlargement and pleomorphism. SQC often shows orangeophilic cells, fiber cells and tadpole cells.
- Adenocarcinoma: Gland-like arrangement and background mucin may help in diagnosis of adenocarcinoma.

BOX 12.21 Differential diagnosis of large cell carcinoma (LCC)

- Sarcomas: Rare; immunostain—vimentin for sarcoma, cytokeratin (CK) for LCC
- Amelanotic melanoma: HMB45 immunostaining.
- Reactive changes: Regular nuclear margin and no significant chromatin abnormality
- Squamous cell carcinoma: Orangeophilic cells, fiber cells and tadpole cells
- Adenocarcinoma: Gland-like arrangement and background mucin

Carcinoid

Carcinoid tumor comprises of a small proportion of primary lung tumors. WHO classified carcinoid as typical carcinoid and atypical carcinoid tumor. The key cytological features are mentioned in **Box 12.22**.

Cytology

Sputum sample of carcinoid tumor is usually paucicellular because the tumor cells rarely shed in the sputum as the overlying mucosa of the tumor remains preserved. The cells are usually seen in bronchial brushing. Cytology smears of the carcinoid tumor usually show dissociated cells. Occasional rosette-like structures may be seen. Individual cells have small to moderate amount of cytoplasm having fine reddish granules (**Figs 12.32** and **12.33**). The nuclei are central in location and relatively monomorphic. Chromatin is finely stippled and gives the characteristic salt and pepper appearance. Nucleoli are usually small and inconspicuous.

Cells of atypical carcinoid show large nuclei with moderate pleomorphism (**Fig. 12.34**). The individual cells have moderate to abundant reddish granular cytoplasm.

Differential Diagnosis

- Small cell carcinoma: See in small cell carcinoma.
- Other lung carcinomas: Cells of bronchioloalveolar carcinoma with monomorphic nuclei may create diagnostic confusion with carcinoid. However, cytological features, such as

BOX 12.22 Carcinoid

- Dissociated cells
- Rosettes
- Monomorphic cells
- Moderate to abundant cytoplasm with red granularity
- Round monomorphic nuclei with salt and pepper chromatin
- Mitosis is uncommon

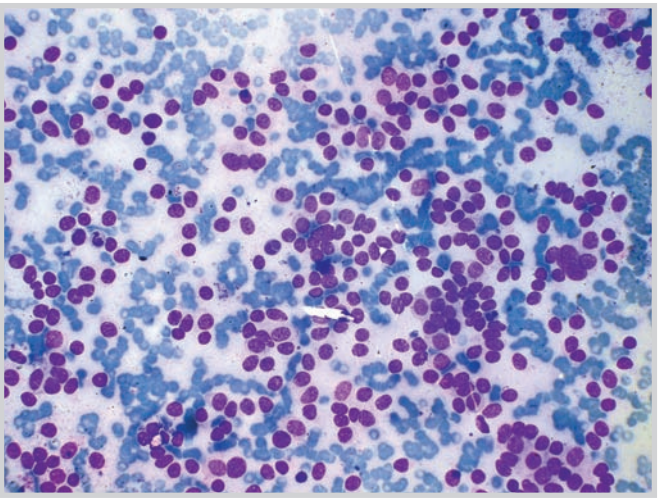

Fig. 12.32: Smears show discrete small round monomorphic cells in carcinoid of lung (May-Grunwald Giemsa stain X MP)

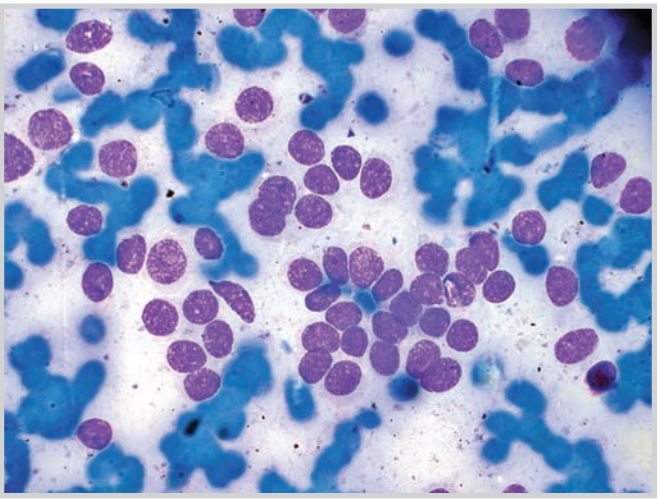

Fig. 12.33: High-powered view of the same showing monomorphic nuclei with stippled chromatin (May-Grunwald Giemsa stain X HP)

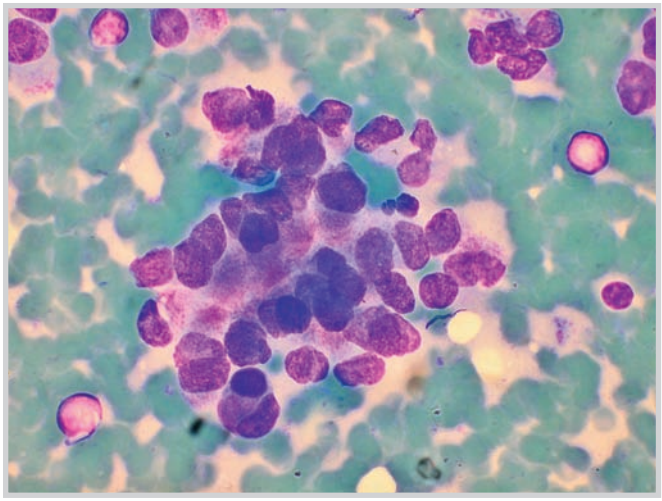

Fig. 12.34: Smear of atypical carcinoid shows cells with moderately pleomorphic nuclei. Cytoplasm shows reddish granulation (May-Grunwald Giemsa stain X HP)

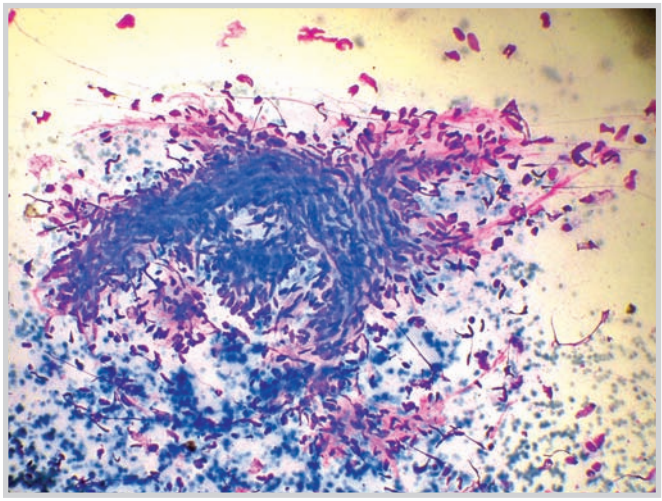

Fig. 12.35: Cytology smear of metastatic leiomyosarcoma in lung showing oval to elongated mildly pleomorphic nuclei (May-Grunwald Giemsa stain X MP)

monolayered sheets, frequent aggregation of cells, cells with vacuolated cytoplasm favor BAC.

Lymphomas

Primary NHL and Hodgkin's lymphoma (HL) are uncommon in the lung. In fact, less than 10 % of extra nodal lymphomas occur in the lung. Primary pulmonary HLs are exceedingly rare. The common primary NHLs of the lung are marginal zone lymphoma and diffuse B cell lymphoma.

Metastatic Malignancies

The lung is the most common site of metastasis, and large varieties of malignancies metastases in the lung (**Fig. 12.35**). However, metastatic carcinomas are uncommonly seen in sputum, bronchial washing or brushing specimen. The most common primary sites of metastatic lung carcinomas are breast, colon, stomach, pancreas, kidney, prostate, liver, male genitalia and female genital tract. This happens because lung is the sole organ that receives the entire blood and lymphatic flow. A thorough clinical history with the detailed knowledge of previous histological diagnosis is needed for the exact diagnosis.

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CHAPTER 13

Gastrointestinal Tract

Chapter Contents

INTRODUCTION

Cytology plays an important role in the diagnosis of various lesions of gastrointestinal tract (GIT) particularly due to rapid improvement of technology of the sampling method and visualization techniques. It is now proved as an efficient technique for the diagnosis of various lesions in GIT. There are several added advantages of exfoliative and endoscopic aspiration cytology in GIT. These are:

- Wide area of the mucosal surface can be covered
- Quick turn over time
- Less invasive technology
- Deeper and stenotic part of the canal can be reached.

SAMPLING TECHNIQUES

The common techniques to collect sample from the gastrointestinal tract are listed in **Box 13.1**.

Endoscopic Brush Cytology

Procurement of the sample under direct visualization of the abnormal mucosa with the help of flexible fiberoptic endoscopy is a major advance in detection of lesions of GIT.[1,2]. This is one of the most common techniques to obtain samples from upper GIT under direct visualization (**Box 13.2**).

Procedure

In endoscopic brushing technology, a flexible fiberoptic endoscope is introduced in the GIT. The flexible nature of the tube allows it to pass through the tortuous food canal. The endoscope contains specific channels for biopsy forceps and brush. The brush is made of nylon and is covered by a Teflon sheath to prevent the loss of cytology material. When the mucosal lesion is visualized, the brush is taken out from the outer Teflon sheath

BOX 13.1 **Sampling techniques in gastrointestinal tract**

- Endoscopic brush cytology
- Salvage cytology
- Endoscopic fine needle aspiration cytology (FNAC)
- Endoscopic ultrasound-guided (EUS) FNAC
- Balloon technique
- FNACbyendoscopicretrogradecholangiopancreatography

BOX 13.2 Brush cytology

Procedure:
With the help of flexible fiberoptic endoscopy, the lesion is visualized and endoscopic brush is taken through the channel of endoscope
Advantages
- Large surface area of the mucosa is covered
- Deeper part of a stenotic canal can be sampled
- Comparatively cost-effective and less invasive

Disadvantages
- Difficult to differentiate severe dysplasia from invasive carcinoma
- Low sensitivity to detect Barrett's esophagus

Comments:
Combined use of biopsy and brush cytology has 100% sensitivity

and gently rubbed on the lesion to get the material. Finally, the brush is retracted into the teflon sheath and is withdrawn from the endoscope. Multiple smears are made by rubbing the brush on the glass slide.

The *major advantages* of brush cytology are: (1) It can procure samples from the large surface area of the mucosa under visual surveillance. As brush cytology covers a large surface area so it is the most suitable technique for detection of infections, (2) brush cytology can be done from the deepest part of a stenotic canal when the usual tissue biopsy forceps cannot enter through the narrowed canal and (3) brush cytology is comparatively less expensive, cost-effective and less invasive technique than other sampling modalities. Brush cytology is complementary to biopsy. Combined use of biopsy and brush cytology increases the sensitivity of detection of malignancy near about 100%.[3] The *major problems* in brush cytology are: (1) It is difficult to differentiate severe dysplasia from invasive carcinoma on cytology and therefore the clinicians may prefer biopsy rather than brush cytology, (2) it is difficult to detect goblet cell metaplasia in esophageal brush and therefore the sensitivity of brush cytology to detect Barrett's esophagus is low.[4]

Salvage Cytology

This is rarely used for diagnosis. However, it is a relatively rapid and easy technique.

Procedure

After obtaining the biopsy, the endoscopic channel is flushed through normal saline and the fluid is collected and processed for smear preparation. The idea of the lavage cytology is to retrieve the material from the endoscopic tube that is dislodged from the external surface of the forceps during withdrawal of the forceps after biopsy.

Endoscopic Fine Needle Aspiration Cytology

The endoscopic FNAC helps in obtaining samples from the lesions located deep in the mucosa.

Procedure

The FNAC needle is introduced through the channel of the fiberoptic endoscope. After localizing the lesion, the needle is introduced moved to and fro in the lesion maintaining negative suction. Finally, the needle is withdrawn and the aspirated material is spread on the glass slide.

Endoscopic FNAC is particularly helpful in submucosal tumors and is complementary to biopsy. The combined use of biopsy, brush cytology and endoscopic FNAC gives 100% sensitivity.[5]

Endoscopic Ultrasound-guided (EUS) FNAC

It is a very useful sampling technique in the diagnosis of the submucosal lesion, such as carcinoid tumor, infiltrative tumor, lymphomas and stromal tumor. EUS helps in localizing along with an assessment of the size and extent of the lesion that is situated underneath the mucosa. EUS guidance helps in better placement of the needle in the submucosal lesion and therefore sensitivity of this technique is high.[6]

Balloon Technique

It is predominantly used in some of the areas of China where the incidence of esophageal malignancy is very high.[7,8] It is an inexpensive and minimally invasive technique. This technique does not need any endoscope. The abrasive balloon technique is used for population screening of the high risk area of esophageal cancer.

A deflated balloon is swallowed by the patient. Within the stomach the balloon is inflated and dragged through the esophageal wall. Ultimately the balloon is deflated at the level of cricoids cartilage and the whole thing is withdrawn and rubbed on a glass slide to make the smear.

Endoscopic Retrograde Cholangiopancreatography

Endoscopic retrograde cholangiopancreatography (ERCP) is a complex technique by which the endoscope is passed through the second part of the duodenum and a plastic catheter is introduced into the ampulla of Vater. Radiographic dye is injected through the catheter to the bile duct and pancreatic ducts and the photographs are taken. The stenotic portion of the bile or pancreatic duct is brushed with the help of a brush introduced through the endoscope.

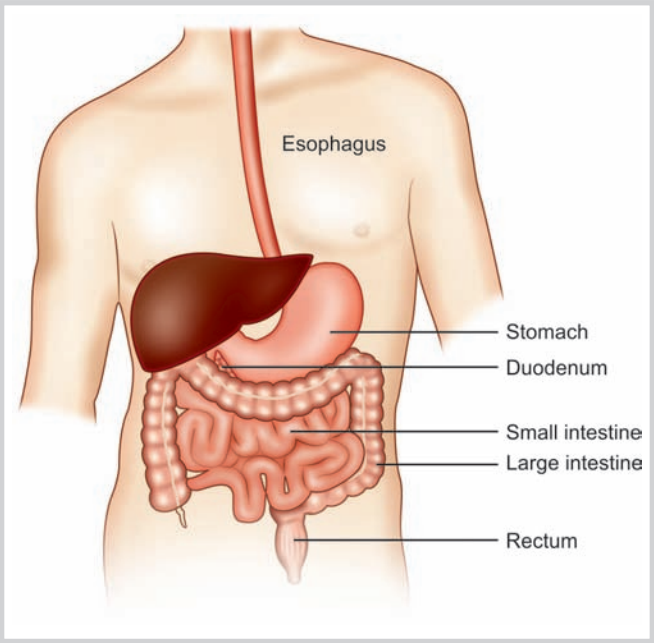


Fig. 13.1: Schematic diagram of gastrointestinal tract

BOX 13.3 Diseases of esophagus

- Infection
  - Fungal: *Candida*
  - Viral: *Herpes*, *Cytomegalovirus*
- Esophagitis
- Radiation and chemotherapy induced changes
- Barrett's esophagitis
- Neoplasm
- Benign: Leiomyoma
- Malignant
  - Squamous cell carcinoma
  - Adenocarcinoma
  - Small cell carcinoma

BOX 13.4 Candidal esophagitis

- Candidal pseudohyphae and budding yeast
- Squamous cells with mild atypia
- Acute inflammatory cells

ESOPHAGUS

Normal Histology

Figure 13.1 shows a schematic diagram of anatomy of GIT. The esophagus is a muscular tube 25 cm long. It has four layers: mucosal, submucosal, muscular and adventitial. Almost the entire esophagus is lined with nonkeratinizing stratified squamous epithelium. The distal 1–2 cm part of the esophagus is lined by simple columnar epithelium.

Immediately under the mucosal lining is the lamina propria which contains loose connective tissue and multiple esophageal cardiac glands. The submucosa of the esophagus contains dense fibroconnective tissue and esophageal glands.

Cytology

Cytological specimen of the esophagus contains squamous epithelial cells. The cells are large, polygonal with abundant eosinophilic cytoplasm and centrally placed nuclei. Nuclear chromatin is fine with inconspicuous nucleoli.

Benign Diseases of Esophagus (Box 13.3)

Infection

Brush cytology is commonly used for detection of various infections of the esophagus. *Candida*, herpes virus and cytomegalovirus are common infections in the esophagus that are diagnosed by brush cytology. These infections are commonly seen in immunocompromised patients.

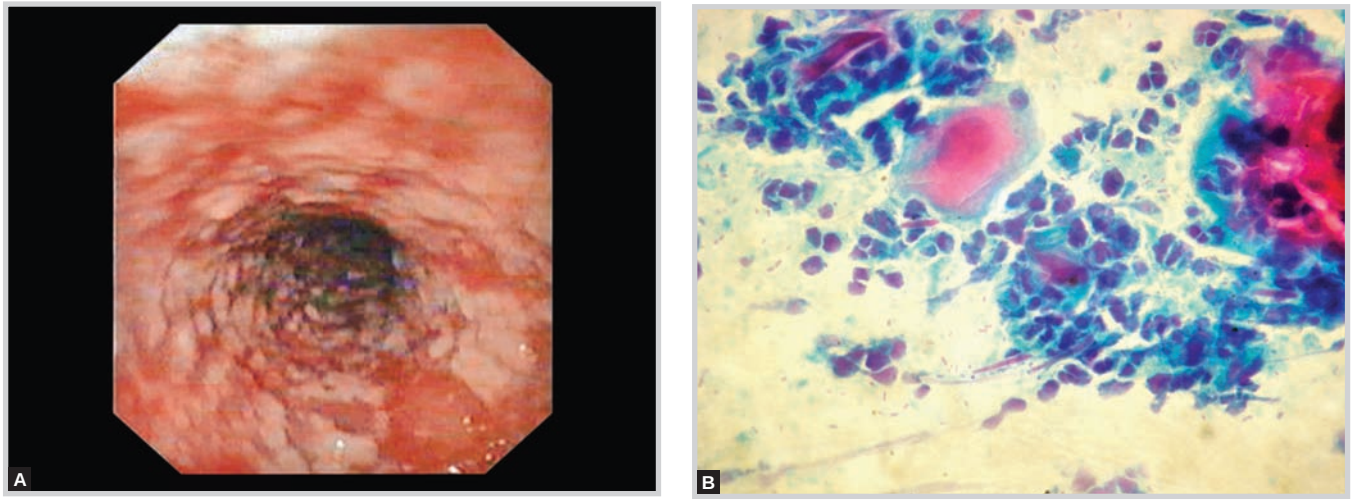
Candida

Candidal esophagitis is the most common fungal infection of the esophagus. It usually occurs in the immunocompromised patient. Whitish pseudomembrane is seen in esophageal mucosa under endoscopic examination (**Fig. 13.2A**). Cytological diagnosis is relatively straightforward (**Box 13.4**). Smear shows many candidal pseudohyphae and yeast form along with squamous cells and polymorphs. Delicate thin elongated and constricted pseudohyphae can be readily evident in the Papanicolaou (PAP) stained smear. Small round spore is also noted by careful examination (**Fig. 13.2B**). The squamous cells may show reactive nuclear atypia. Candidal contamination from oral area is usually not a problem as the endoscopic brush is covered by a sheath.

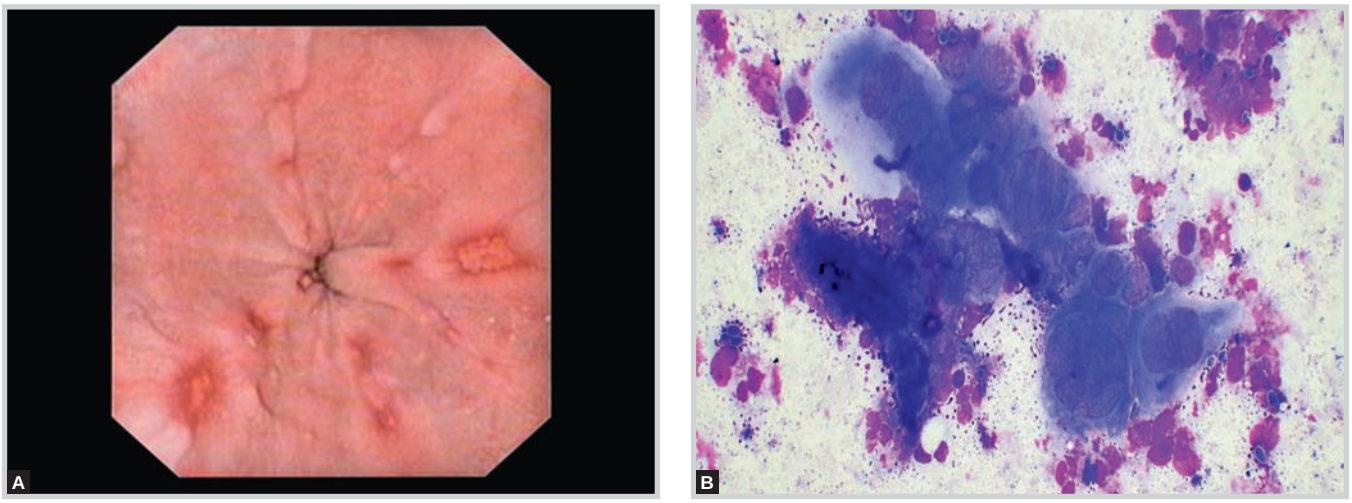
Herpes Simplex Viral Esophagitis

Herpes simplex virus infection is the most common cause of viral esophagitis. It usually occurs in immunocompromised patients, diabetic patients, carcinoma cases and patients who receive radiation and chemotherapy. Endoscopic examination classically shows multiple small reddish shallow ulcers with sharp margins in the distal part of the esophagus (**Fig. 13.3A**). Cytological features of herpetic esophagitis are similar as seen in other parts of the body (**Box 13.5**).

The cytology smear shows many multinucleated giant cells. Nuclei are closely spaced and often show characteristic nuclear molding (**Fig. 13.3B**). Individual cells show large intranuclear inclusions with a clear halo at the periphery and thick nuclear membrane. In many cells, nuclear chromatin looks like refractile ground-glass. In addition, the squamous cells often show nuclear atypia. The atypical squamous cells may be mistaken as malignant cell. However, the N/C ratio of the cell is not increased and nuclear margin is regular.



Figs 13.2A and B: (A) Endoscopic examination shows whitish patches in *Candidia* infection of esophagus; (B) Candidal spores and pseudohyphae in a case of candidal esophagitis (Papanicolaou's stain X HP)
Source Fig. 13.2 A: S Sinha, Additional Professor of Gastroenterology, PGIMER, Chandigarh, India



Figs 13.3A and B: Endoscopic examination shows multiple small reddish shallow ulcers with sharp margin in herpetic esophagitis; (B) Multinulceated cells with nuclear molding in a case of herpetic esophagitis (May-Grünwald-Giemsa stain X HP)
Source Fig 13.3A: S Sinha, Additional Professor of Gastroenterology, PGIMER, Chandigarh, India

BOX 13.5 Herpetic esophagitis

- Risk factors: Immunocompromised patient, cancer, radiation and chemotherapy
- Endoscopic features: Multiple small reddish shallow ulcers with sharp margin
- Cytology
  - Multinucleated giant cells
  - Molded nuclei
  - Large intranuclear inclusions
  - Clear halo around the inclusion with thick margin
  - Homogenized ground-glass chromatin
  - Reactive atypia in squamous cells

Cytomegalovirus Infection

Cytomegalovirus (CMV) infection in the esophagus is uncommon and it usually occurs in immunocompromised patients (**Box 13.6**). The endoscopic and clinical features are nonspecific. CMV mainly infects the endothelial cells, fibroblasts and glandular elements. Squamous cells in the mucosa are not involved by CMV. Therefore, endoscopic biopsy rather than brush is the choice of diagnostic modality.

Cytology smears of the esophageal brush rarely show the characteristic CMV infected cell. The infected cell shows cytomegaly with preserved N/C ratio. Nuclei show large intranuclear inclusion with a peripheral clear halo and margination of chromatin on the inner nuclear membrane. Small satellite basophilic cytoplasmic inclusions are also noted.

BOX 13.6 Cytomegalovirus esophagitis

- Endoscopic feature: Nonspecific
- Infects the endothelial cells and fibroblasts and glandular elements
- The choice of diagnostic modality: endoscopic biopsy
- Immunocompromised patient is affected
- Cytology
  - Enlarged cell
  - Both cell and nuclei are enlarged
  - Large amphophilic intranuclear inclusion
  - Peripheral clear halo and margination of chromatin
  - Small satellite basophilic cytoplasmic inclusions

Esophagitis

Esophagitis may be noted in various conditions such as reflux esophagitis, corrosive ingestion, alcohol, drugs etc. (**Box 13.7**). Endoscopy shows erosion and ulceration.

Cytology smear from the brush sample shows cohesive clusters of cells with flowing or streaming pattern of cells giving "school of fish" appearance. The individual squamous cells show nuclear enlargement and prominent nucleoli. However, N/C ratio is well preserved and nuclear margin is regular. Nuclear chromatin is fine. In addition, background of the smear shows inflammatory cells, increased mitosis and atypical stromal cells.

Differential Diagnosis

The atypical squamous cells may be mistaken as malignant cells. The distinguishing cytological features are preserved N/C ratio, regular nuclear margin and fine nuclear chromatin (**Box 13.8**).

Radiation and Chemotherapy Induced Changes

Both radiation and chemotherapeutic drugs show similar changes (**Box 13.9**). Endoscopy shows reddish, friable and hemorrhagic areas.

The cytology smear of brushing sample shows discrete atypical cells with enlarged nuclei. The N/C ratio of the cell is well preserved. Individual cells have frayed cytoplasmic border with intracytoplasmic vacuoles. Nuclei show smudgy chromatin with irregular outline. Occasional multinucleated cells are also seen.

Barrett's Esophagus

Barrett's esophagus (BE) is an acquired condition where the lower part of the squamous epithelial lining is replaced by columnar epithelial cells with intestinal metaplasia containing goblet cells (**Box 13.10**). It is thought as a protective mechanism to save the lower distal part of the esophagus from the recurrent gastric reflux. It is considered as a preneoplastic condition and the majority of the esophageal adenocarcinoma develops from the Barrett's esophagitis.

BOX 13.7 Esophagitis/repair changes

- Causes: Reflux esophagitis, corrosive ingestion, alcohol, drug ingestion
- Endoscopy: Hyperemia, erosion, ulceration
- Cytology
  - Reactive squamous cells with "school of fish" appearance, i.e. streaming pattern of cells
  - Enlarged nuclei
  - Preserved N/C ratio
  - Regular nuclear margin
  - Evenly distributed chromatin
  - Prominent nucleoli
  - Mitosis
  - Inflammatory cells and atypical stromal cells

Differential diagnosis: Carcinoma

BOX 13.8 Distinguishing features between repair and malignancy

Favorable points for malignancy

- Many discrete cells
- Over crowded cells and loss of polarity
- Enlarged nuclei
- High N/C ratio
- Irregular and thickened nuclear margin
- Irregular-shaped multiple nucleoli
- Hyperchromatic coarse nuclei
- Abnormal mitotic figures
- Tumor diathesis

BOX 13.9 Radiation and chemotherapy effects

- Endoscopy: Reddish, friable and hemorrhagic areas
- Cytology
  - Enlarged cells
  - Frayed cytoplasmic border
  - Vacuolated cytoplasm
  - Nuclear enlargement
  - Preserved N/C ratio
  - Smudged chromatin
  - Multinucleation

Clinical symptoms of the patients are heartburn and dysphagia. Endoscopic examination shows characteristic salmon-pink color with an admixture of intestine-like epithelium.

Cytology smear of BE shows cohesive sheets of epithelial cells with monomorphic nuclei. The outline of the clusters is regular. The nuclear polarity of the cell is well-maintained. Identification of goblet cell is essential for the diagnosis of BE. The cells have abundant vacuolated cytoplasm. The size of the vacuole is three times more than the nucleus. Cytological diagnosis of BE has low sensitivity and specificity. This may be due to sampling error.[9]

BOX 13.10 Barrett's esophagus

Definition: Lower part of the squamous epithelial lining is replaced by columnar epithelial cells with intestinal metaplasia
- Premalignant condition
- Clinical features: Heartburn and dysphagia
- Endoscopy: Characteristic salmon-pink color with admixture of intestine-like epithelium
- Cytology
  - Cohesive sheets of epithelial cells
  - Regular border of the cluster
  - No nuclear overlapping
  - Goblet cells
    - Cells with large cytoplasmic vacuoles three times the size of nucleus

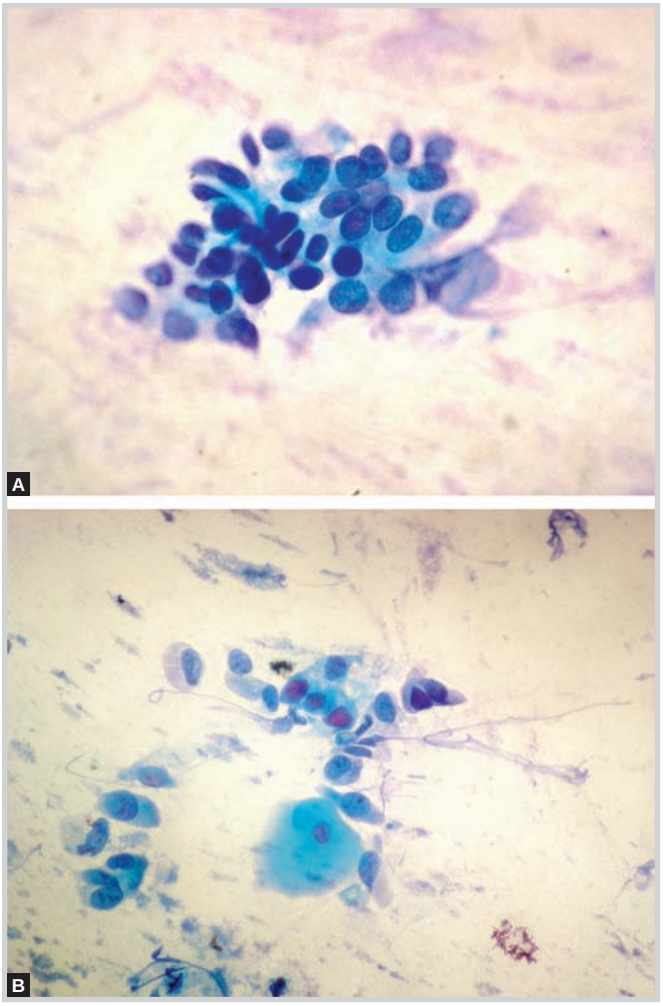
BOX 13.11 Dysplasia in Barrett's esophagus

- Small clusters of cells with irregular outline
- Nuclear overlapping and loss of polarity
- Columnar cells
- Nuclear enlargement, pleomorphism, thickened nuclear membrane
- Hyperchromasia with prominent nucleoli
- Clean background

Dysplasia in Barrett's Esophagus

Almost all esophageal adenocarcinoma develop form Barrett's esophagus.[10] A series of dysplastic changes in the mucosal epithelium occurs before the development of adenocarcinoma. Therefore, the diagnosis of the degree of dysplasia is important in a setting of BE.

Cytological features of dysplasia in BE depend on the degree of dysplastic changes (**Box 13.11**). The smears of high grade dysplasia show loose cohesive groups of cells with irregular border. There is significant nuclear crowding, overlapping and loss of polarity. The individual cells are columnar in appearance with enlarged hyperchromatic nuclei. The nuclear margin is irregular and thickened. Nucleoli are large. The atypical cells are sparse in number compared to adenocarcinoma (**Figs 13.4A and B**). The background of the smear is clean and no tumor diathesis is seen.



Figs 13.4A and B: (A) Dysplasia in esophagus. Smear shows cohesive cluster of cells with mild nuclear atypia (Papanicolaou's stain X MP); (B) Dysplasia in esophagus. Smear shows dissociated cells, moderate amount of cytoplasm and mild nuclear atypia (Papanicolaou's stain X MP)

Squamous Cell Carcinoma

Squamous cell carcinoma (SQC) is the most common malignancy of the esophagus and includes more than 85% of esophageal malignancies. The patients are usually in 6th decade. The usual clinical symptoms are dysphagia and loss of weight. The common risk factors of SQC of the esophagus are cigarette smoking, alcohol abuse, vitamin A, C, E and riboflavin deficiency.

The cytological features (**Box 13.12**) of SQC are similar to that of other organs. Smears show small clusters and discrete cells. In case of keratinizing SQC, the individual cells are polyhedral with well-defined cytoplasmic border (**Figs 13.5** to **13.7**). The cytoplasm shows orangeophilic appearance. Nuclei are hyperchromatic, moderately pleomorphic nuclei with inconspicuous nucleoli. Occasional keratin pearls are also noted. In nonkeratinizing SQC, smear shows round to oval cells with relatively scanty cytoplasm. Nucleus shows moderate pleomorphism, hyperchromasia and clumped irregular chromatin. Poorly-differentiated carcinoma may show prominent nucleoli.

Adenocarcinoma

Adenocarcinoma of the esophagus arises from the lower part of the esophagus. EUS-guided FNAC is a preferable sampling modality than brush cytology for the detection of adenocarcinoma of the esophagus. Smears show the glandular arrangement of cells, loose clusters and discrete cells. Individual cells show a moderate amount of vacuolated cytoplasm. Nuclei are moderately pleomorphic with prominent nucleoli (**Box 13.13**) (**Figs 13.8A and B**).

BOX 13.12 Squamous cell carcinoma

- Discrete and loose cluster of cells
- Polyhedral-shaped
- Orangeophilic cytoplasm
- Hyperchromatic nuclei
- Moderate nuclear pleomorphism
- Inconspicuous nucleoli
- Irregular clumped chromatin
- Keratin pearls
- Background necrosis

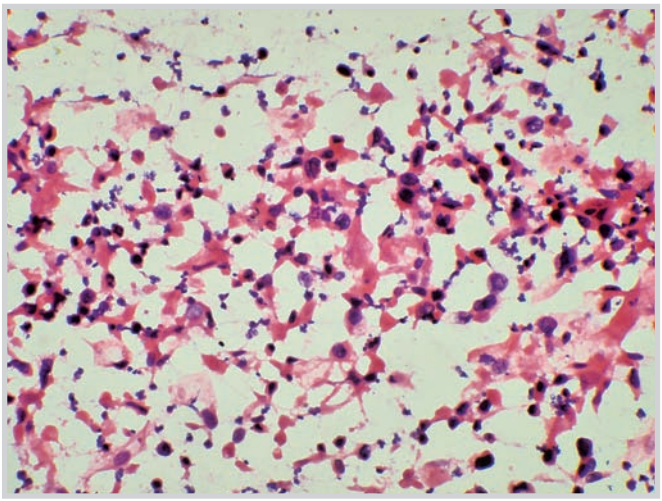


Fig. 13.5: Malignant cells with orangeophilic cytoplasm and central hyperchromatic nuclei in squamous cell carcinoma (Papanicolaou's stain X MP)

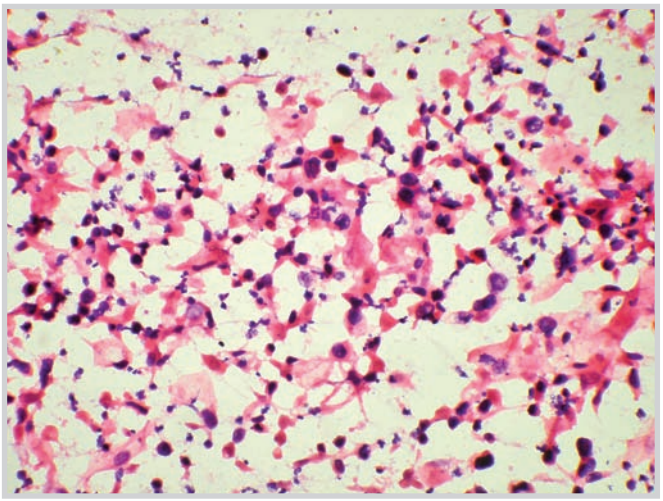


Fig. 13.7: Fiber cells in squamous cell carcinoma (Papanicolaou's stain X HP)

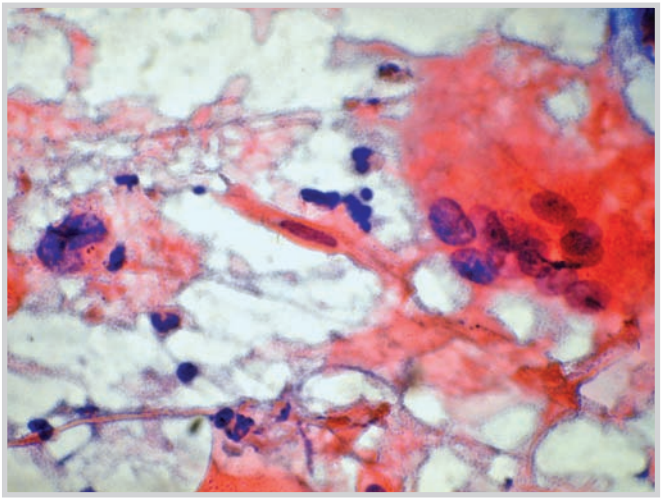


Fig. 13.6: Many discrete oval to polyhedral malignant cells with centrally placed nuclei having moderate nuclear pleomorphism in squamous cell carcinoma (Papanicolaou's stain X MP)

BOX 13.13 Adenocarcinoma of esophagus

- Occurs in lower part of esophagus
- Rich cellularity
- Background: Tumor diathesis
- Arrangement
  - Multiple gland-like arrangement of cells, clusters of cells
  - Overlapping or crowded arrangement of cells
  - Peripheral feathering of cell
- Nuclei
  - Enlarged
  - Pleomorphic
  - Irregular margin
  - Prominent nucleoli
- Cytoplasm: Moderately vacuolated

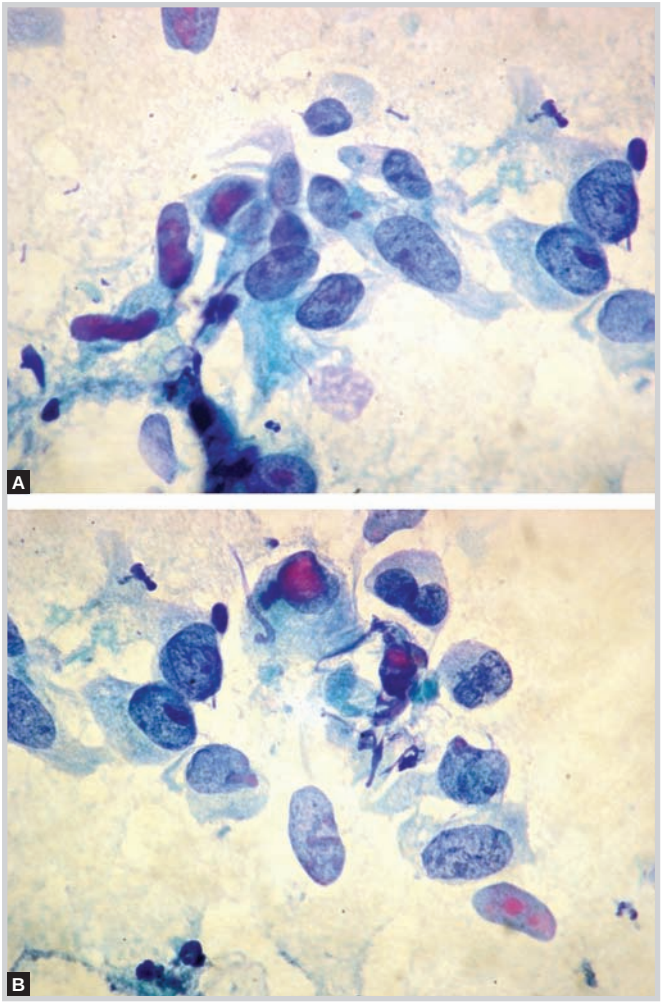
Small Cell Carcinoma

Small cell carcinoma (SCC) (**Box 13.14**) is an uncommon tumor of the esophagus. SCC accounts for less than 5% of esophageal carcinoma. It is a highly aggressive tumor.

Smears show predominantly discrete cells with scanty cytoplasm. Nuclei are small hyperchromatic. Chromatin is finely granular and gives a salt and pepper appearance. Nuclear molding is also noted.

BOX 13.14 Small cell carcinoma

- Discrete cells
- Small cells slightly larger than lymphocytes
- Scanty cytoplasm
- Nuclear molding present
- Hyperchromatic nuclei
- Salt and pepper chromatin
- Absent nucleoli



Figs 13.8A and B: (A) Moderately pleomorphic nuclei with prominent nucleoli in a case of adenocarcinoma in esophageal brush cytology (Papanicolaou's stain X HP); (B) Malignant cells with moderate amount of cytoplasm and moderately pleomorphic nuclei with prominent nucleoli in a case of adenocarcinoma in esophageal brush cytology (Papanicolaou's stain X HP)

STOMACH

Anatomy

The stomach has four regions: cardiac, fundus, body and pyloric region.

Cardiac: It is the narrow region at the gastroesophageal junction and frequently filled with gas.

Fundus: This is the small dome-shaped part situated left of the esophagus.

Body: This is the main part of the stomach.

Pylorus: This is the narrow constricted part in between the body and duodenum.

The whole stomach has multiple longitudinal folds of mucosa and submucosa known as rugae. Rugae permit distension of the stomach when food is ingested and in distended stomach, the rugae disappear. The epithelial lining of the stomach invaginates within the mucosa forming gastric pits. Gastric glands open in the gastric pits.

Histology

The gastric mucosa is lined by simple columnar epithelium. The surface and neck of the glands are lined by mucus-secreting columnar cells. The mucosal glands produce thick mucin that protects the stomach from the irritant substances and acid digestion. In addition, the glands in the fundus and body of the stomach also contain parietal and chief cells. The parietal cells manufacture hydrochloric acid and intrinsic factors. The parietal cells are round in shape with eosinophilic cytoplasm and basally placed nuclei. The chief cells produce various enzymes, such as pepsinogen, renin and gastric lipase. These are columnar looking cells with basophilic cytoplasm and basally placed nuclei. In addition of these cells, scattered endocrine cells are also present.

Cytology

Mucus Secreting Cells

The mucus cells are usually present in small clusters and in flat sheets. The cells are usually in monolayer and polarity of the cells is well maintained. Dispersed single cells are infrequent in brush cytology. The individual cells are tall, columnar in appearance with abundant vacuolated cytoplasm (**Fig. 13.9**). The nuclei are central in position, round and monomorphic with fine nuclear chromatin and inconspicuous nucleoli. Occasionally, nuclear protrusion may be noted.

Chief Cells

These are cuboidal cells with numerous coarse basophilic cytoplasmic granules. Nuclei are central in position and round in shape.

Parietal Cells

These are round cells with marked vacuolization of the cytoplasm. Cytoplasmic eosinophilic granules are seen. Nuclei are centrally placed having coarse chromatin.

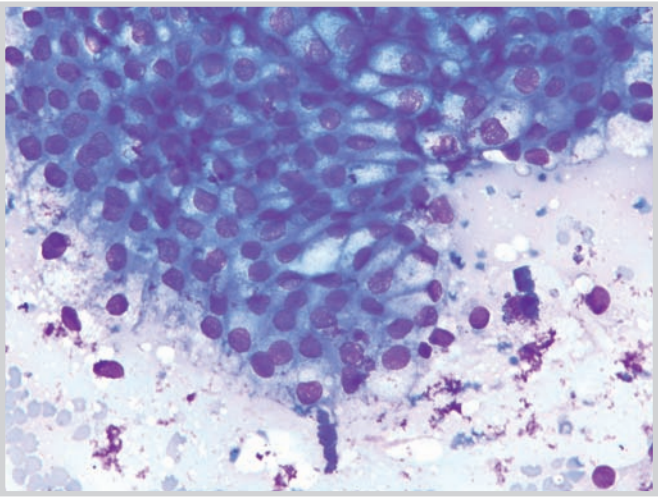


Fig. 13.9: Normal columnar epithelium of gastric mucosa (May-Grünwald-Giemsa stain X HP)

BOX 13.15 Acute gastritis

- Etiological causes: Aspirin ingestion, alcohol, heavy smoking, chemotherapy, burn or trauma, etc.
- Cytology
  - Polymorphs
  - Degenerated cells
  - Cohesive sheets of glandular cells
  - Reactive changes of the epithelial cells

Benign Diseases of Stomach

Acute Gastritis

Acute gastritis (**Box 13.15**) is caused by various factors, such as aspirin ingestion, alcohol ingestion, heavy smoking, chemotherapy, burn, trauma, etc. Cytology is not done for diagnosis of acute gastritis. The cytology smear of acute gastritis shows cohesive sheets of glandular cells (**Box 13.15**). The cells may show reactive atypia. Nuclei of the cells may be enlarged; however, N/C ratio is well preserved. In addition, the background of the smear shows abundant polymorphs.

Chronic Gastritis

Cytology smears of chronic gastritis show multiple cohesive sheets of glandular cells with reactive atypia. Nuclei may show a mild increase of the N/C ratio with relatively enlarged nuclei, nuclear pleomorphism and hyperchromasia. Nucleoli may be prominent. In case of gastric ulcer, there may be necrotic debris and inflammatory cells. Marked nuclear atypia of the gastric epithelial cells may mimic malignancy. At times, it is very difficult to distinguish reactive atypia and carcinoma. The absence of significant nuclear pleomorphism and regular nuclear margin are in favor of reactive atypia.

BOX 13.16 *Helicobacter pylori*

- Associated with chronic gastritis and peptic ulcer disease
- May-Grünwald-Giemsa staining is best
- 2–3 micron long, curved, or spiral-shaped structure
- Risk factor for gastric carcinoma and non-Hodgkin's lymphoma of stomach

Helicobacter pylori

Helicobacter pylori is usually associated with chronic gastritis and peptic ulcer disease (**Box 13.16**). It can be easily detected by gastric crush smear, brush cytology or imprint smear. Brush cytology is preferable to demonstrate this organism as it covers the wide surface area of the mucosal membrane. The organisms are best demonstrated in the May-Grünwald-Giemsa (MGG) staining. The organisms are mainly seen in the thin mucus as 2–3 micron long, curved or spiral-shaped structure. In addition, the cytology smears also show sheets of epithelial cells with mild reactive atypia.

Benign Tumor

Gastric Polyp

Most of the gastric polyps are hyperplastic polyps with no risk factor for development of carcinoma. These polyps are small and may be single or multiple. Cytology smears show multiple clusters of epithelial cells with mild reactive atypia.

Malignant Tumors

Gastric Adenocarcinoma

Adenocarcinoma is the most common malignant tumor of the stomach. A steady decline in the incidence of gastric carcinoma has been noted worldwide in the last few decades. Several strong associations of gastric carcinoma are known, such as *Helicobacter pylori* infection, smoking, intake of large amount of smoked fish, pickled vegetables and low socioeconomic setting. Early gastric carcinoma may not produce any symptoms and some of the patients may have only dyspeptic symptoms. Absence of any symptoms is responsible for detection of gastric carcinoma in advanced stages. In advanced stages, it may cause pain, vomiting, hematemesis and melena. Patients may also have severe anemia.

There are two types of gastric adenocarcinoma, intestinal and diffuse (gastric) type.

Cytology (Box 13.17)

Intestinal Carcinoma: The cytology smear shows loose cluster and discrete cells. The cells are in three-dimensional clusters. Individual cells show marked nuclear enlargement and high N/C ratio. Nuclei are moderately pleomorphic and hyperchromatic with irregular nuclear margin (**Fig. 13.10**). Prominent nucleoli and frequent mitosis are also noted.

BOX 13.17 Gastric adenocarcinoma

Intestinal type
- Single cells, loose cohesive clusters of cells
- Columnar or cuboidal cells
- Marked nuclear pleomorphism
- Enlarged nuclei with high N/C ratio
- Hyperchromatic nuclei
- Irregular nuclear margin
- Prominent nucleoli

Diffuse or gastric type
- Predominantly single cells and occasional loose clusters
- Round cells with abundant vacuolated cytoplasm
- Crescent-shaped nucleus pushed to the periphery
- Hyperchromatic nucleus
- Prominent large nucleoli
- Pool of mucin

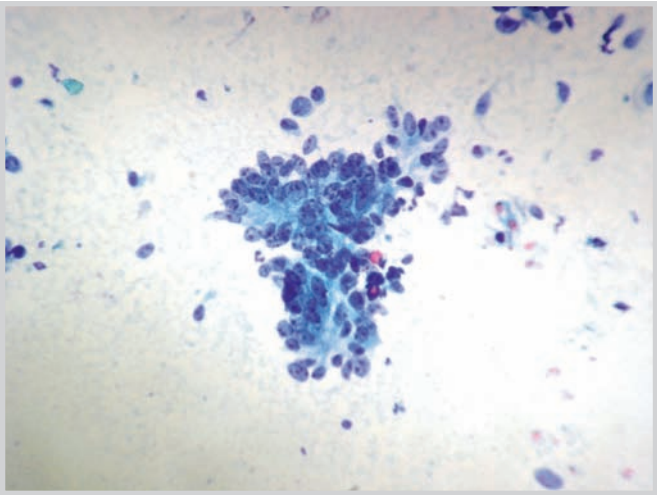


Fig. 13.10: Cluster of malignant cells with moderate nuclear enlargement and pleomorphism in a case of intestinal type adenocarcinoma of stomach (Papanicolaou's stain X MP)

Diffuse or Gastric Type: The diffuse type of gastric cancer is mainly made of the signet ring type of cells. The cells are mainly discrete with occasional loose small clusters. Individual cells are round to oval with the abundant vacuolated cytoplasm. Nuclei are eccentric in position and crescent in shape. Nuclear margin is irregular. Nucleoli are usually prominent (**Fig. 13.11**).

Differential Diagnosis

Signet ring cells are often difficult to distinguish from macrophages. The signet ring cells are positive for pancytokeratin and epithelial membrane antigen (EMA) and negative for CD61.

Gastrointestinal Stromal Tumor

Gastrointestinal stromal tumor (GIST) commonly affects the older individuals. GIST is most commonly seen in the stomach followed by the small intestine and rectum. The origin of the tumor is from the interstitial cells of Cajal or primitive stem cells. These cells are also known as gastrointestinal pacemaker cells. The behavior of the tumor depends on its size and mitotic activity. Tumors with less than 5 cm size are usually benign in nature. Tumors with more than 5 cm diameter in size but less than 5 mitosis per 50 high power field are designated as "uncertain malignant potential". Histological grade of GIST depends on the mitotic activity. Tumors with more than 10 mitosis per 10 high power field are labeled as high grade tumors. Whereas, tumors with 1–5 mitosis per 10 high power field are considered as low grade.

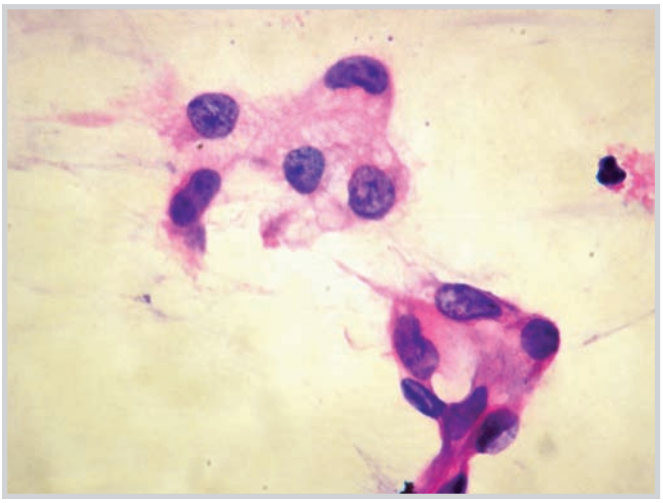


Fig. 13.11: Signet ring type adenocarcinoma of stomach showing malignant cells with large vacuolated cytoplasm and nuclei are pushed to the periphery (Hematoxylin and Eosin stain X HP)

The patient presents with hematemesis and pain abdomen. Brush cytology of the stomach is not helpful for diagnosis of this tumor unless the tumor ulcerates the mucosa. EUS-FNA is the best way to diagnose the submucosal GIST of the stomach. Percutaneous FNAC under USG guidance may also be helpful in relatively larger tumor.

Cytology

Cytology smear shows loose cohesive clusters, small fascicles and dissociated spindle-shaped cells (**Box 13.18**). The individual cells show scanty to moderate cytoplasm and oval to elongated spindle shaped nuclei (**Fig. 13.12**). Nuclei show mild-to-moderate pleomorphism, coarse granular chromatin and inconspicuous to absent nucleoli. CD117 (C-Kit) positivity is essential for diagnosis of GIST. In addition, the cells are also positive for CD34 (membranous pattern of positivity) and focally positive for smooth muscle actin.

Diagnostic Difficulties

Other Spindle Cell Soft Tissue Tumor: Gastrointestinal stromal tumor always has a differential diagnosis of other spindle cell mesenchymal tumor, such as leiomyoma, schwannoma and their malignant counterpart. At times, it is difficult to differentiate GIST form these tumors on cytology alone. A panel of immunostaining may be helpful in this context (**Table 13.1**).

Carcinoid Tumors (Neuroendocrine Tumor)

The term neuroendocrine tumor is presently replaced by carcinoid tumor. These are potentially malignant tumors and often associated with hormone production with recognizable clinical syndromes. The appendix is the most common site of origin of carcinoid in GIT followed by the rectum, small intestine and stomach.[11] In stomach, it is most frequently associated with atrophic gastritis. In nonatrophic gastritis, it may occur from Zollinger-Ellison syndrome. Gastric carcinoid is infrequent and account for less than 1% of all gastric malignancies.[12]

BOX 13.18 Gastrointestinal stromal tumor

Cytology
- Clusters, fascicles and also dissociated cells
- Spindle cells with scanty to moderate cytoplasm
- Spindle-shaped nuclei with mild pleomorphism
- Coarse granular chromatin, indistinct nucleoli

Immunocytochemistry: Positive for CD117 (C-kit) and CD34

Histological grade:
- High grade tumors: Tumors with more than 10 mitosis per 10 high power field
- Low grade: Tumors with 1–5 mitosis per 10 high power field

Cyogenetics:

Malignant GIST usually shows
- Losses in chromosomes 14 and 22
- Gains and amplification of 3q, 8q, 5p and Xp region
- Mutations of the c-kit gene

Cytology

The cytology smear shows predominantly discrete, loose cohesive clusters, trabecular and rosette-like arrangement of cells. The individual cells are monomorphic round cells with scanty to moderate reddish granular cytoplasm (**Fig. 13.13**). Many spindle-shaped cells may be seen. The nuclei are round monomorphic, with salt and pepper chromatin and inconspicuous nucleoli (**Box 13.19**).

Diagnostic Difficulties

- *Adenocarcinoma:* Carcinoid tumor with acinar-like arrangement of cells may simulate adenocarcinoma. The individual cell morphology of carcinoid such as reddish granular cytoplasm and monomorphic nuclei with salt and pepper chromatin are helpful in differentiating from adenocarcinoma. Immunocytochemistry may be helpful in certain situations. Carcinoid cells are positive for chromogranin and synaptophysin.

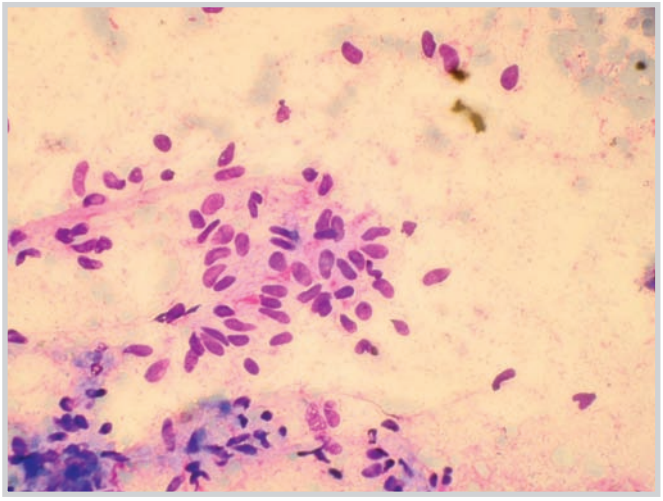


Fig. 13.12: Oval to elongated spindle cells with mild nuclear pleomorphism in gastrointestinal stromal tumor (May-Grünwald-Giemsa stain X HP)

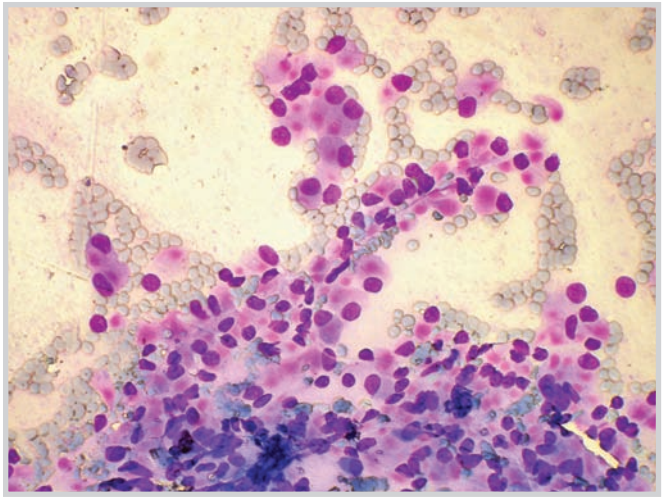


Fig. 13.13: Discrete tumor cells with monomorphic nuclei and moderate amount of pinkish granular cytoplasm in carcinoid of the stomach (May-Grünwald-Giemsa stain X HP)

TABLE 13.1: Immunostaining of different spindle cell tumors

Lesion	*C-Kit (CD117)*	*CD34*	*Desmin*	*S-100*	*SMA*	*CK*
GIST	+	+ (60%)	-	+ (occasional)	+ (30–40%)	-
Smooth muscle tumor	-	+	+	-	+	-
Schwannoma	-	-	-	+	-	-
Spindle cell carcinoma						+

Abbreviations: GIST, Gastroinetstinal stromal tumor; SMA: Smooth muscle actin; CK, Cytokeratins

BOX 13.19 Carcinoid tumor

- All carcinoid tumors are low grade malignant tumors
- Frequencies: Appendix, rectum, small intestine (ileum, jejunum, duodenum), stomach
- Gastric carcinoid rare (less than1%)
- Clinical symptoms: Classical symptoms rare,
  - Stomach: Hematemesis, pain
  - Carcinoid symptoms in intestine: Diarrhea, flushing, bronchospasm
- Cytology
- Singly and in loose aggregates
- Monomorphic round cells with scanty to moderate reddish granular cytoplasm
- Eccentric round nuclei, minimal pleomorphism
- Salt and pepper chromatin
- Small inconspicuous nucleoli
- Immunocytochemistry: Positive for chromogranin, synaptophysin

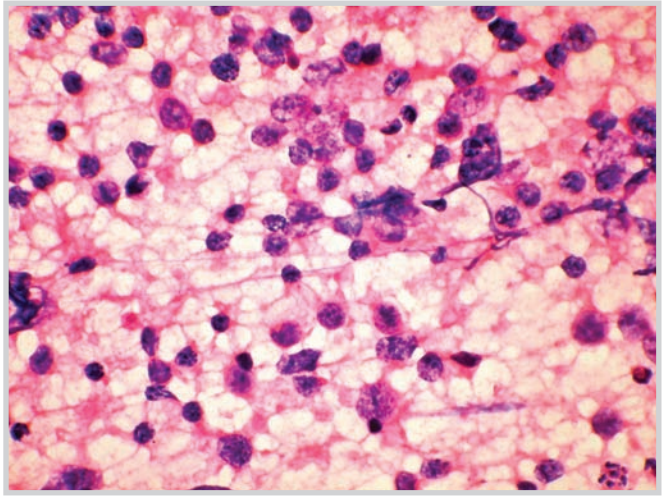


Fig. 13.14: Discrete immature lymphoid cells with fine nuclear chromatin in a case of non-Hodgkin's lymphoma of stomach (Papanicolaou's stain X HP)

TABLE 13.2: Differential diagnosis of mucosa-associated lymphoid tissue lymphoma (MALToma)

Features	*Lymphoma*	*Small cell carcinoma*	*Carcinoid*	*Poorly-differentiated carcinoma*
Cell dissociation	Predominantly discrete cells	Discrete and small clusters	Discrete and small clusters	Small clusters
Lymphoglandular bodies	Present	Absent	Absent	Absent
Cytoplasm	Scanty	Scanty	Scanty to moderate	Moderate
Cytoplasmic granularity	Absent	Absent	Present	Absent
Nuclear molding	Absent	Present	Absent	Absent
Nuclear threading	Absent	Present	Absent	Absent
Nucleoli	Absent	Absent	Absent	Present
Immunocytochemistry				
LCA	Positive	Negative	Negative	Negative
CK	Negative	Negative	Positive	Positive
Chromogranin	Negative	Positive	Positive	Negative

Abbreviations: LCA, Leukocyte common antigen; CK, Cytokeratin

- *Small cell carcinoma:* Predominant discrete small cells in carcinoid tumor may often pose diagnostic difficulty with small cell carcinoma. Nuclear threading, nuclear molding and condensed hyperchromatic nuclei favor a diagnosis of small cell carcinoma.

Non-Hodgkin Lymphoma

Primary gastric lymphoma arises from the stomach and contiguous group of lymph nodes. It is considered as primary lymphoma of the stomach if the main tumor bulk is located in the stomach only. The majority of the gastric lymphomas are high grade B cell lymphoma and some of these lymphomas have evoloved from a mucosa associated lymphoma (MALT, B cell type). GIT is the most common extranodal site of the non-Hodgkin's lymphoma (NHL). Lymphoma accounts about 10% of all gastric malignancies. There is a high association of *Helicobacter pylori* and low grade MALT.[13]

Cytology smears show discrete cells with round nuclei. The nuclei are relatively monomorphic with scanty cytoplasm. Nuclear chromatin is condensed with inconspicuous nucleoli. Background shows lymphoglandular bodies (**Fig. 13.14**).

Brush cytology is helpful in the diagnosis of diffuse large B cell lymphoma. The cells are discrete with a relatively larger nucleus showing mild to moderate pleomorphism. Nuclei show coarse clumped chromatin, irregular nuclear membrane and prominent nucleoli.

Differential Diagnosis

- *Small cell carcinoma:* Small cell carcinoma often comes in the differential diagnosis of low grade MALT (**Table 13.2**). Nuclear threading and molding are two important differentiating

cytological features of small cell carcinoma. In addition, the background lymphoglandular bodies are absent in small cell carcinoma.

- *Poorly-differentiated carcinoma:* Poorly-differentiated carcinoma often poses diagnostic difficulty with diffuse large cell NHL. The cytology smears show loose clusters of cells, moderate cytoplasm, nuclear pleomorphism and absence of lymphoglandular bodies.
- *Carcinoid:* Cytology smears of carcinoid show cells with moderate reddish granular cytoplasm and absence of lymphoglandular bodies.

SMALL INTESTINE

Anatomy and Histology

The small intestine is 7m long tube. The surface area of the intestine is formed by transverse folds, multiple villi, microvilli and crypts of Lieberkühn. The intestinal mucosa is composed of simple columnar epithelium, the lamina propria and the muscularis mucosa. The surface mucosal lining of the intestine is made of surface absorptive cells, goblet cells and diffuse neuroendocrine system (DNES) cells.

Surface Absorptive Cells

These are tall columnar cells with a basally placed round to oval nuclei. The cells have a low N/C ratio. The apical surface of the cell is covered by brush border.

Goblet Cells

Goblet cells are intermingled with surface absorptive cells. They are mucus producing cells and contain large vacuoles in the cytoplasm. Duodenum has the smallest number of goblet cells. The number of goblet cells increases from duodenum to ileum.

Neuroendocrine Cells

The mucosal surface of the small intestine also shows variable number of neuroendocrine cells that secrete various endocrine substances.

Cytology

Cytology smear shows flat monolayer sheets of cells. The cells are often arranged as honeycomb manner. The individual cells are tall columnar in appearance with round nuclei having a low N/C ratio. Nuclei are monomorphic with fine chromatin and small nucleoli. The goblet cells have large vacuolated cytoplasm and round small nuclei.

Infections

Giardia lamblia

Giardia lamblia is a common parasite in the duodenum. The organism is noted either by endoscopic biopsy or by FNAC. *Giardia lamblia* is usually located over the crypts of the mucosal surface of the duodenum. This is a 12–15 micron diameter, pear-shaped binucleated organism with four pairs of flagella.

Cryptosporidium

Cryptosporidium is usually seen in immunocompromised patients. The majority of the patient presents with watery diarrhea and fever. The protozoa are present on the luminal surface or within the glandular cells and are best seen in MGG stained smear. They are 2–3 micron sized round basophilic organism.

Tuberculosis

Tuberculosis is commonly noted in the small intestine. Percutaneous FNAC is the usual sampling technique in mass lesions of the abdomen. Cytology smears show multiple epithelioid cell granulomas, giant cells and necrosis. Ziehl-Neelsen stain shows acid fast bacilli.[14]

Adenocarcinoma

Adenocarcinoma of duodenum occurs more frequently than other malignancies. Cytology smears of adenocarcinoma show clusters and discrete cells with occasional glandular arrangement. The individual cells are columnar in appearance with moderate amount of vacuolated cytoplasm. Nuclei are moderately pleomorphic with prominent nucleoli.

Lymphoma

Non-Hodgkin's lymphomas are more frequent in small intestine than carcinomas. The patient usually presents with pain abdomen and weakness. MALT is a type of low grade NHL. However, there may be transformation of low grade NHL to high grade NHL. Most of these MALTomas are B cell type. The cells are small to medium sized with scanty cytoplasm. Nuclei are round with fine chromatin. Plasma cell differentiation is common in intestinal MALTomas. There may be a good number of transformed centroblats or immunolblasts in MALtomas (**Figs 13.15** and **13.16**)

LARGE INTESTINE

Anatomy and Histology

Large intestine includes cecum, colon, rectum and anal canal. Colon has no villi. Rectal and colonic mucosa are lined by a single or double layer of columnar cell and abundant goblet cells.

Cytology smear shows multiple small clusters and single columnar cells. The cells are columnar in shape with distinct cell margin and round monomorphic nuclei. Many goblet cells are also seen.

Acute and Chronic Inflammation

Large intestine is affected by a wide variety of infections causing acute or chronic inflammation. Cytological sampling is rarely

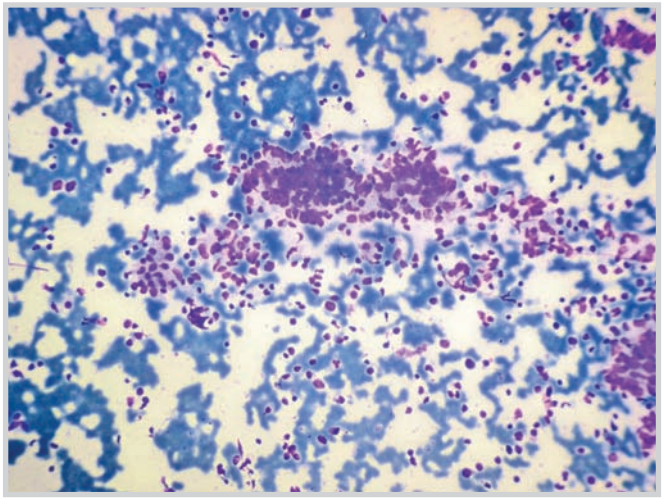


Fig. 13.15: Ultrasonography-guided FNAC smear showing discrete immature lymphoid cells in a small intestinal lymphoma (May-Grünwald-Giemsa stain X MP)

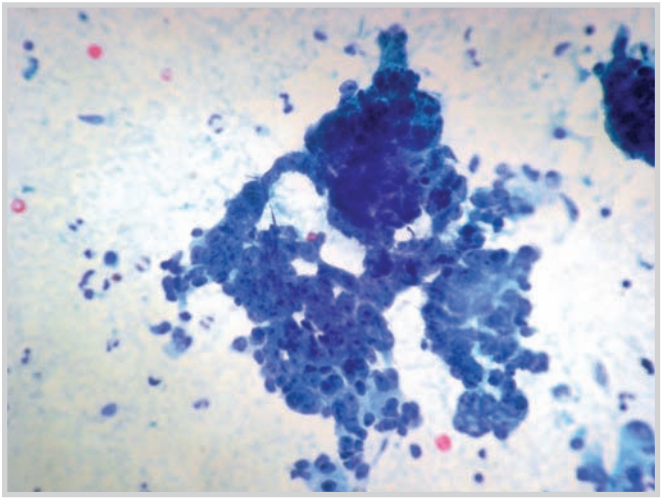


Fig. 13.17: Cluster of malignant cells with scanty cytoplasm and moderately pleomorphic nuclei having prominent nucleoli in crush smear of colonic adenocarcinoma (Papanicolaou's stain X HP)

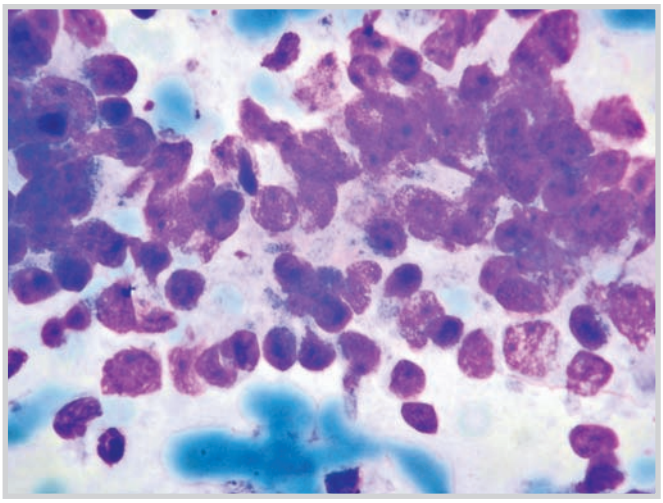


Fig. 13.16: High powered view showing large cells with fine nuclear chromatin and single prominent nucleoli (May-Grünwald-Giemsa stain X HP)

done for diagnosis of acute or chronic colitis. Colonoscopic examination can be performed easily along with colonic biopsy. Biopsy is more informative regarding dysplasia and presence of foci of infection.

Colorectal adenocarcinoma usually occurs in the 6th decade of life. The patient presents with sudden alteration of bowel habits, rectal bleeding and anemia. Brush cytology is helpful in diagnosis of adenocarcinoma. The cytomorphology of adenocarcinoma is similar to that of adenocarcinoma of duodenum. A cytology smear of well and moderately differentiated adenocarcinoma shows multiple clusters of columnar cells with gland-like arrangement at places. The cells are columnar with moderately pleomorphic nuclei and prominent nucleoli (**Fig. 13.17**).

Anal Cytology

Major portion of the anal canal is lined by stratified squamous epithelium except the proximal part of the anal canal adjacent to the rectum that is lined by cuboidal to columnar epithelium.

Human papilloma virus (HPV) infection of the anal canal and subsequent development of dysplastic lesions followed by squamous cell carcinoma has promoted the screening of the anal canal region to detect dysplastic lesions. The incidence of SQC in the anal canal is about 35 per 100,000 population in homosexual men. This is quite high incidence rate and is comparable to the incidence of cervical cancer in women before the era of PAP screening. Both SQC of cervix of women and anal canal in men has the same etiological link of infection of high risk HPV infection.[15-17] Screening of anal canal PAP test in high risk population for HPV-related changes and dysplasia may have some effect in early diagnosis of dysplastic lesions.[18]

The anal PAP test is done by introducing a moistened swab blindly into the anal canal and then rotating firmly on the wall of the anal canal. Finally, the smear is made by pressing the swab directly on the slide.

The cytologic reporting criteria of the anal PAP test are similar to that of Bethesda System Classification of cervical cytology. However, the sensitivity of the anal PAP test is relatively lower than cervical cytology.

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CHAPTER 14

Cerebrospinal Fluid

Chapter Contents

INTRODUCTION

Cerebrospinal fluid (CSF) is normally found in the subarachnoid space. It helps in the nourishment and protection of the brain and spinal cord. CSF is formed in the ventricles by choroid plexus and bathes the brain and spinal cord. It circulates through the subarachnoid space of the spinal meninges. Unlike effusion fluid, CSF is normally produced in the ventricles and normal lining cells of the subarachnoid and pial meninges do not shed into CSF. Examination of CSF provides valuable information for diagnosis of various infective conditions, leukemic infiltration, metastatic tumor and some primary central nervous system (CNS) tumor. Indications of cytologic examination of CSF are highlighted in **Box 14.1**.

BOX 14.1 Indications of cerebrospinal fluid examination

- Infections
  - Bacterial meningitis
  - Fungal meningitis
- Metastatic tumors
- Leukemia and lymphoma infiltration
- Primary central nervous system tumor rarely
- Ventricular disease
  - Trauma
  - Infraction
- Demyleinating diseases
  - Guilain Barre syndrome
  - Multiple scleroisis

ANATOMY

Choroid plexus is the complex folded membrane of the ependymal lining of the ventricles that generate CSF. CSF is continuously produced by the choroid plexus and circulates through the ventricles to subarachnoid space after passing through the ventricles via the midline foramen of Magendie (**Fig. 14.1**). Finally, it comes to the central canal of the spinal cord. CSF is absorbed in the arachnoid granulations and comes into the venous system.

CYTOLOGY

Cerebrospinal fluid is a clear fluid and contains mainly water (90%), glucose, protein and occasional lymphocytes. The lining cells of meninges such as pia and arachnoid very rarely shed into the CSF.

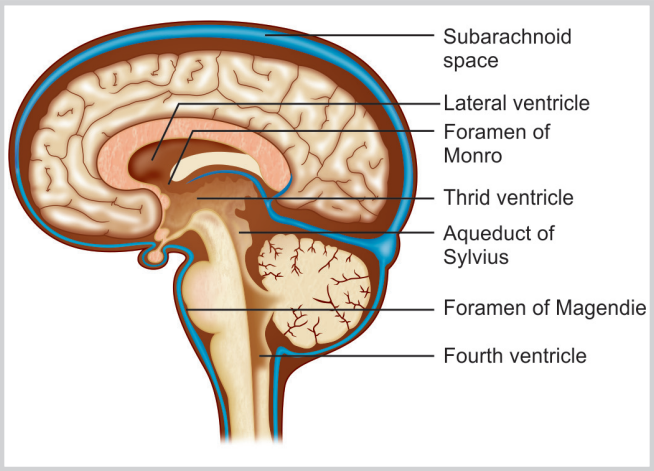


Fig. 14.1: Schematic view of circulation of cerebrospinal fluid in the central nervous system

Sampling of CSF

Cerebrospinal fluid is collected by lumbar puncture technique and about 4–5 mL CSF is withdrawn during spinal tap. The portion of CSF should be collected in a clean plastic tube for cytological examination and should be sent immediately to the laboratory. No preservative is recommended for CSF collection. Author recommends immediate processing of CSF.

Laboratory Technique

Cerebrospinal fluid is processed by cytocentrifugation technique. At least two smears are made: one for Papanicolaou (PAP) stain and the other for May-Grünwald-Giemsa (MGG) stain. If necessary, multiple smears can be made for special stain. One portion of CSF can be kept for flow cytometry.

Normal Cells

Cerebrospinal fluid usually contains very scanty cellularity. It contains only a few lymphocytes. Mature lymphocytes have small round nuclei with condensed chromatin and scanty cytoplasm. Reactive lymphocytes have relatively more cytoplasm and enlarged nuclei having small nucleoli. There may be sparse monocytes. The monocytes have relatively more cytoplasm and kidney-shaped nuclei.

Neutrophils may also be present in CSF sample. Neutrophil count increases due to infection, particularly bacterial infection. RBCs are generally not seen in CSF sample. However, during the surgical procedure RBCs may come to CSF. The presence of excess RBCs in CSF indicates trauma or hemorrhage. Rarely, ependymal cells are seen in CSF sample. These cells have moderate cytoplasm with round to oval centrally placed nuclei.

Cerebrospinal fluid may occasionally be contaminated by starch granules from the gloves of the surgeon.

Infective Conditions

Acute Meningitis

Acute bacterial meningitis is caused by many gram-positive and gram-negative organisms. Smears show large numbers of polymorphonuclear leukocytes and occasional lymphocytes (**Box 14.2**). The overall cell count in CSF may be as high as 30–40 thousand per cubic ml. In addition, bacterial organisms can be demonstrated by Gram stain. Microbiological culture is the diagnostic test for exact identification of bacterial organisms.

Viral Meningitis

Viral meningitis is caused by mumps, herpes, enteroviruses and cytomegaloviruses (CMV). Most of the time the course of infection is benign and self recovery occurs except viral encephalomyelitis which has fatal course. Cytology smears show increased cell count (1,000–3,000 per cubic ml). The majority of the cells are lymphocytes. In addition transformed atypical lymphocytes are also seen (**Box 14.3**). The cells have relatively more cytoplasm and nuclei with opened up chromatin and single prominent nucleoli.

Tuberculous Meningitis

Tuberculosis of the meninges produces nonspecific changes. Cell count in CSF increases and predominant cell population are lymphocytes along with occasional plasma cells and histiocytes. The presence of multinucleated giant cell is a nonspecific finding.

AIDS Meningitis

- AIDS encephalopathy occurs directly or indirectly from infection of cells within the brain or spinal cord by the AIDS virus. In this condition, we get nonspecific chronic inflammatory infiltrate.
- Opportunistic infections are most frequently produced by *Cryptococcus neoformans, Toxoplasma gondii, Mycobacteria, Candida*, and viruses such as CMV.
- CNS lymphoma may be seen in AIDS patients.

BOX 14.2 **Acute meningitis**

- Abundant polymorphs
- Bactria: may or may not be present

BOX 14.3 **Viral meningitis**

- Lymphocytes
- Atypical lymphocytes

BOX 14.4 **Cryptococcal meningitis**

- Immunocompromised patient
- Polymorphonuclear leukocytes
- Yeast form of fungus: Round-shaped, 5–10 micron diameter structure that often shows narrow based budding
- Capsule of the fungus stains with alcian blue and mucicarmine

Fungal Meningitis

Cryptococcal meningitis: *Cryptococcus neofromans* is the most frequent fungal infection of CSF. It may occur both in healthy and also immunocompromised patient (**Box 14.4**). The smear shows poymorphonuclear leukocytes and a yeast form of fungus. The yeast is round-shaped, 5–10 micron diameter structure that often shows narrow based budding. Cryptococci has a mucopolysaccharide capsule that stains with alcian blue and mucicarmine.

Demyelinating diseases: Multiple my○eloma: It produces the normal cell count in CSF. T cell population increases. T helper/suppressor ratio decreases.[1]

In case of Guillain-Barré syndrome, the cytology smear of CSF is acellular. Occasionally, CSF may show lymphocytic response.

Neoplasm

Metastatic solid tumor: Any malignancy may metastasize in the leptomeninges and may be detected in CSF. However, leukemia, lymphoma and carcinomas are more frequently seen in CSF. Majority of metastatic carcinomas in CSF are adenocarcinoma from lung, breast and gastrointestinal tract. Most of time meningeal carcinoma is associated with parenchymal involvement of brain.[2,3] Malignant cells come to CSF by direct extension from the parenchyma of the brain or by the extension of the tumor from bone to subdural space or by following the tracks of cranial or spinal nerve.

Proper clinical history of primary tumor and histological type of primary tumor are very important. Occult cases of breast or GIT tumor are very uncommon. However, occasionally pulmonary tumor may be small and asymptomatic. Cytological examination of CSF is very important to document the leptomeningeal involvement of the tumor.

LUNG CARCINOMA

Metastatic adenocarcinoma of the lung is more common than small cell carcinoma and squamous cell carcinoma. Cytology smears of CSF shows clusters and occasional dissociated cells with moderate to abundant cytoplasm. Nuclei are round with moderately pleomorphic having prominent nucleoli.

Small carcinoma of lung shows discrete cells. Cells may show linear arrangement with nuclear molding. The cytoplasm of the cells is scanty. Nuclei show small hyperchromatic nuclei.

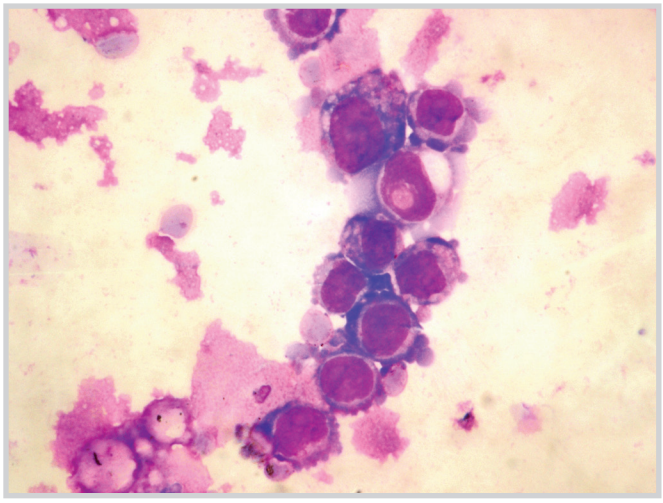


Fig. 14.2: Cells of infiltrating duct carcinoma of breast in cerebrospinal fluid. Smear shows enlarged cells with pleomorphic nuclei (May-Grünwald-Giemsa stain X HP)

BREAST CARCINOMA

Carcinoma of breast uncommonly metastasizes to CSF. Smears show discrete round cells with moderate cytoplasm. Nuclei are round with moderately pleomorphic nuclei and prominent nucleoli (**Fig. 14.2**).

Cytology smears of lobular carcinoma show discrete cells with occasional rows of cells. Cells are smaller in size with scanty cytoplasm and round relatively monomorphic nuclei. Signet ring type of cell is also seen.

MELANOMA

Melanoma may metastasize from the skin or other organs. The cells are large with moderately pleomorphic nuclei having prominent macro-nucleoli. The cells may also contain melanin pigment in the cytoplasm.

LEUKEMIA

Leukemic infiltration of leptomeninges may be noted without any involvement of brain parenchyma. Many patients with leukemic infiltration are asymptomatic. Therefore, the routine CSF examination is very useful for diagnosis of leukemic infiltration.

Acute Lymphoblastic Leukemia

Acute lymphoblastic leukemia (ALL) is the most common leukemia in childhood age period. CNS relapse of ALL occurs in more than 50 % cases. Periodic examination of CSF is required for detection of ALL infiltration in CSF. CSF of ALL infiltration usually shows excess lymphoid cells (**Box 14.5**). The cells are round with scanty deep blue cytoplasm and round nuclei with fine nuclear chromatin (**Figs 14.3** and **14.4**). Nucleoli are large and prominent. In addition, nuclei often show large nipple-like protrusion.

BOX 14.5 Acute lymphoblastic leukemia

- The most common childhood leukemia
- Excess lymphoid cells
- Round cells with scanty cytoplasm
- Nuclear chromatin fine
- Nipple-like nuclear protrusion
- Large prominent nucleoli

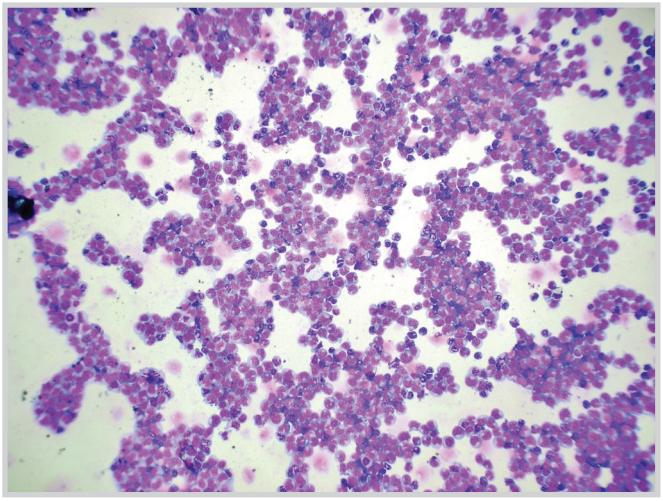


Fig. 14.3: Cerebrospinal fluid smear of acute lymphoblastic leukemia showing round cells with scanty deep blue cytoplasm and round nuclei with fine nuclear chromatin (May-Grünwald-Giemsa stain X MP)

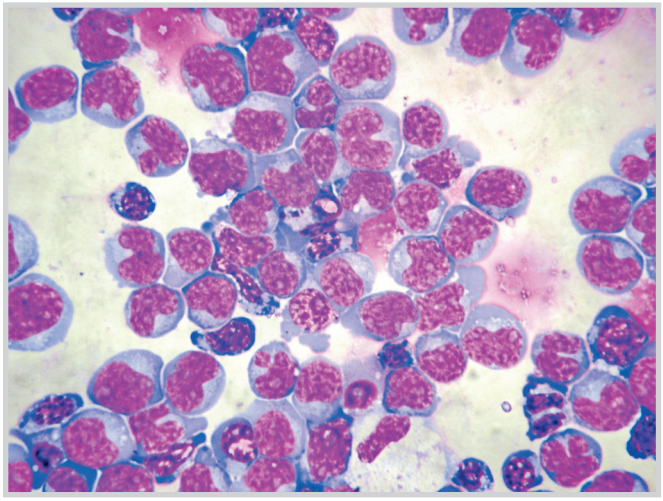


Fig. 14.4: High powered view showing better cell morphology of acute lymphoblastic leukemia in cerebrospinal fluid. Nuclei are enlarged with reticular nuclear chromatin (May-Grünwald-Giemsa stain X OI)

Interpretation Problems

- *Contamination with blood*: Cytology smear of CSF may be contaminated by blasts from the peripheral blood. Therefore, adequate precaution should be taken before the interpretation of such smear.
- *Too few cells*: At times, CSF may show scanty cells with occasional blasts. It is difficult to decide whether this patient should be treated as CNS relapse or not. If CSF contains more than 10 cells per cubic mL, then these cases should be treated as CNS relapse.[4]

Acute Myeloblastic Leukemia

Acute myeloblastic leukemia (AML) commonly occurs in adulthood. The incidence of CNS infiltration in AML is much lower than ALL. It may be probably due to the lower survival period of AML. All subtypes of AML infiltrates in the leptomeninges and this may occur at the time of initial presentation or at relapse. The morphology of blasts is similar to that of blood (**Box 14.6**). The cells are large and round with irregular nuclear margin. Nuclear chromatin is fine. Multiple prominent nucleoli are seen. In addition, the cytoplasm of the cells shows azurophilic granules. Auer rods are the linear slender structure in the cytoplasm formed by condensation of azurophilic granules. The presence of characteristic Auer rods is pathognomonic of myeloblast.

BOX 14.6 Acute myeloblastic leukemia (AML)

- Common in adults
- Cerebrospinal fluid involvement is less than acute lymphoblastic leukemia
- Blasts
  - Relatively large cells
  - Irregular nuclear margin
  - Fine chromatin
  - Multiple prominent nucleoli
  - Auer rods may be seen

Chronic Lymphocytic Leukemia

Leptomeningeal involvement of chronic lymphocytic leukemia (CLL) is very much uncommon. It is usually very difficult to differentiate small mature lymphocytes from the cells of CLL. Immunocytochemistry may be helpful in selected cases.

Chronic Myeloid Leukemia

Infiltration by chronic myeloid leukemia (CML) is also extremely uncommon. CSF smears in CML infiltration shows polymorphous population of cells consisting of myelocytes, metamyelocytes,

promyelocytes and polymorphs. It is essential to eliminate the possibility of contamination by blood in such cases.

LYMPHOMAS

Leptomeningeal involvement of lymphoma is similar to that of leukemia. Lymphoma predominantly involves leptomeninges without any parenchymal involvement. About one tenth of NHL involves CNS at the time of presentation or during the course of the disease. Some specific types of lymphoma such as diffuse large B cell lymphoma, Burkitt's and lymphoblastic lymphomas commonly affect leptomeninges (**Fig. 14.5**).[5] Most of the lymphomas in CSF are B cell NHL.

Adequate clinical information, particularly the history of NHL and type of NHL are very helpful in diagnosis. In difficult cases, immunocytochemistry or flow cytometry may be helpful. It has been noted that flow cytometry along with cytological examination, increases the sensitivity of diagnosis of NHL in CSF.[6]

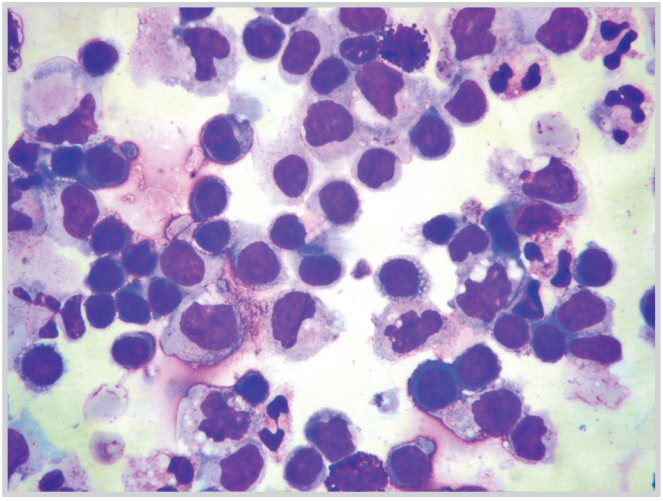
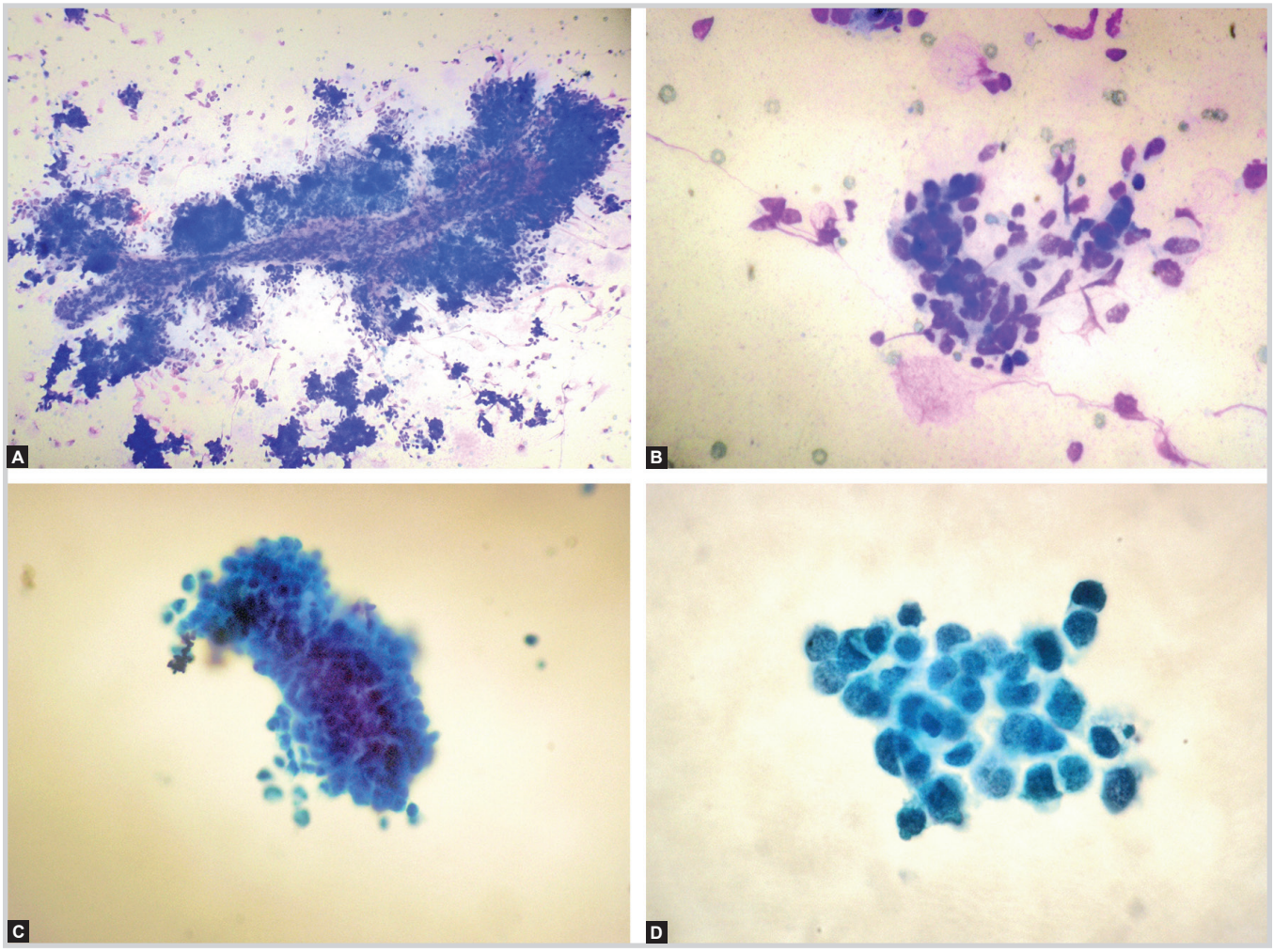


Fig. 14.5: Cerebrospinal fluid smear shows infiltration by a known case of non-Hodgkin's lymphoma (May-Grünwald-Giemsa stain X OI)



Figs 14.6A to D: (A) Perivascular arrangement of cells in ependymoma of cerebrospinal fluid (May-Grünwald-Giemsa stain X LP); (B) Round to oval cells with mildly pleomorphic nuclei having scanty cytoplasm in ependymoma (May-Grünwald-Giemsa stain X MP); (C) Tight cluster of small cells in ependymoma (Papanicolaou's stain X MP); (D) Cells with mildly pleomorphic nuclei in ependymoma (Papanicolaou's stain X HP)

Primary CNS Lymphoma

Primary CNS lymphoma accounts for 10–15% of all primary CNS tumors. Diffuse large B cell NHL is the most common type of Primary CNS lymphoma.[7] In most of the cases, brain parenchyma is involved without leptomeningeal involvement. The diagnosis of primary CNS lymphoma is difficult in CSF examination. Flow cytometric immunophenotyping may be helpful to demonstrate monoclonality of the cells.

OTHER PRIMARY CNS TUMOR

Primary neoplasm of the CNS is rarely noted in CSF except medulloblastoma. CSF is not examined for the investigation primary CNS tumor.

Medulloblastoma

It is a childhood tumor and may invade the CSF through ventricles.[8] Cytology smears show small clusters and dissociated cells (**Box 14.7**). The individual cells have scanty cytoplasm and round to oval hyperchromatic nuclei having prominent nucleoli. Nuclear molding may be seen. The cells are positive for synaptophysisn and negative for glial fibrillary acidic protein.

OTHER BRAIN TUMORS

Among the other primary neoplasms of the brain, germinoma and ependymoma may rarely shed into the CSF (**Figs 14.6A to D**). The cells of ependymoma are usually arranged in small clusters, perivascularly and discretely. Individual cells are small, round with scanty cytoplasm and relatively monomorphic nuclei.

BOX 14.7 Medulloblastoma

- Small clusters and discrete cells
- Round cells with scanty cytoplasm
- Hyperchromatic round nuclei
- Nuclear molding
- Prominent nucleoli

BOX 14.8 Diagnostic problems

Major problems
- Low cellularity in cerebrospinal fluid (CSF)
- Rapid degeneration of cells due to low sugar
- Lower volume of sample
- Malignancy in CSF are difficult to confirm histologically
- Difficult to obtain additional sample

DIAGNOSTIC ACCURACY

Sensitivity of CSF examination for detection of malignancy is 54–60%.[9-11] However, the detection rate of malignancy in CSF increases if multiple samples are examined, the volume of CSF is more and the extent of leptomeningeal involvement is diffuse and extensive. The major problems in CSF examination are relatively low cellularity in CSF, rapid degeneration of cells due to low sugar, low volume of samples examined, difficult to confirm histologically and difficult to obtain an additional sample (**Box 14.8**).

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SECTION 3

Laboratory Techniques in Cytology

CHAPTER 15

Basic Technique and Approach to Fine Needle Aspiration Cytology

Chapter Contents

INTRODUCTION

Fine needle aspiration cytology (FNAC) is now an essential and integral part of cytology. It is also known as fine needle aspiration biopsy (FNAB). In fact, FNAC procedure can be done in any organ of the body or any tissue or swelling.

ADVANTAGES

Fine needle aspiration cytology has several advantages over tissue biopsy (**Box 15.1**).[1] This procedure can be done anywhere in the laboratory, in outpatient department or in the hospital. No special precaution is needed for FNAC of a superficial mass. A patient can be called in the FNAC clinic or outpatient department and within just 1 hour the report can be delivered. This is a cost-effective and rapid technique with high sensitivity and specificity. FNAC is comparable or even better than the frozen section in certain situations such as thyroid or breast lesions. This technique provides significant economical relief in developing countries where health care cost is important. FNAC report provides a great psychological relief to the patient. It can convince the patient that the lesion is benign or infectious in origin. In case of malignancy, it can convince the patient to have an urgent treatment. Nowadays, with the help of ancillary technique on FNAC material, exact diagnosis is possible in most of the lesions. Therefore, FNAC can avoid tissue biopsy in many situations.

BOX 15.1 Advantages of FNAC

- Simple technique
- Office procedure
- Rapid
- Economical
- Wide area of sampling
- High sensitivity and specificity
- Avoids further tissue biopsy in case of inoperable malignancy or infective conditions
- Almost all the ancillary techniques are possible from FNAC material

LIMITATIONS

Fine needle aspiration cytology has certain limitations over tissue biopsy such as loss of architecture of tissue and inability to interpret capsular or vascular invasion (**Box 15.2**). In many conditions, complex tissue architecture is helpful in diagnosis such as soft tissue tumors or skin adnexal lesions. The exact classification of such lesions is difficult in FNAC. Follicular pattern of lymphoma is important in diagnosis of follicular lymphoma. It is difficult to diagnose follicular lymphoma on FNAC material alone. Another challenge in FNAC is to predict capsular or lymphovascular invasion. Similarly, the diagnosis of

BOX 15.2 Limitations of FNAC over tissue biopsy

- Loss of tissue architecture
- Capsular invasion and lymphovascular invasions not possible to predict
- Not possible to comment on in situ versus invasive carcinoma
- Training is needed for accurate interpretation

TABLE 15.1: Exfoliative cytology versus FNAC

Features	*FNAC*	*Exfoliative*
Cellular pattern	Important to examine	Not so important
Cellularity	High cellularity	Diagnostic cells are usually low
Nuclear morphology	Well-preserved	Less well-preserved
Nuclear detail	Less important	Very important

"in situ" or invasive carcinoma is not always possible in FNAC material. Moreover, interpretation of FNAC sample is a bit different than tissue biopsy and considerable skill is needed to interpret FNAC material.[2]

In fact, FNAC interpretation is also different than exfoliative cytology. Unlike exfoliative cytology, interpretation of FNAC needs more importance on the overall cellular pattern (**Table 15.1**).

COMPLICATIONS

Fine needle aspiration cytology of superficial masses are almost free of any complication (**Box 15.3**). Minor hematoma may develop if the area is not compressed after FNAC procedure. Anaphylactic reaction may occur due to accidental rupture of hydatid cyst during FNAC procedure. However, even after FNAC of hydatid cyst this complication is rare unless there is spillage of the cyst fluid in the adjacent tissue.

Complications, if any, of the FNAC procedure mainly occurs in the deeper mass lesion or if the thick bore needle is used. In fact, the incidence of complication is indirectly proportional to the diameter of the aspiration needle.[3,4] Surgical emphysema may occur in lung FNAC. However, in our experience most of such conditions can be managed conservatively. The spillage of tumor through the needle tract is not true if fine bore needle is used.[5] Truly speaking, FNAC can safely be used even in ovarian cyst if a thin needle is used and proper precautions are taken. Rarely, FNAC needle may cause rupture of an aneurysmal vessel or spleen. This can be avoided if proper precautions are taken. In case of the spleen, gentle suction should be done and too much movement in different directions should be avoided. Occasionally, biliary peritonitis and intestinal perforations have been reported[6] and this can be avoided if thin needle is used for FNAC.

BOX 15.3 Complications of FNAC

- Minor hematoma
- Surgical emphysema
- Accidental rupture of aneurysmal vessel or spleen
- Anaphylaxis in hydatid cyst
- Biliary peritonitis and bowel perforation
- Infraction of lymph node and thyroid
- Needle tract seedling of cancer cells (if thick bore needle is used)

BOX 15.4 Contraindications of FNAC

- Hemophilia or gross coagulation disorders
- Aneurysmal dilatation of blood vessels
- Hydatid cyst

CONTRAINDICATIONS

There are no absolute contraindications of FNAC. However, one should be careful to do FNAC in coagulation disorders particularly hemophilia (**Box 15.4**). FNAC should better be avoided in such conditions. In case of severe thrombocytopenia, FNAC may cause hematoma and it should better be avoided in vascular organs in such disorders. In deep seated lesions, a team approach with radiologist, surgeons, physicians and cytologists is needed.

EQUIPMENT

Following equipments are needed for the FNAC procedure to do:

- Pistol handle to hold the syringe (**Fig. 15.1**): The pistol handle is made up of metal and is fit to hold the syringe properly. It also helps to give suction during the FNAC procedure. Its main advantage is that the other hand of the operator remains free to hold the swelling
- Syringe: Depending on the size of the pistol handle 10 or 20 cc disposable plastic syringe is used
- Needle: Ordinary disposable hypodermic needle of 22–27 gauge is used for FNAC. The size of the needle depends on the situation. Large bore needle is especially helpful for hard and fibrotic lesions or cyst with viscous material. On the other hand thin bore needle is used for small lymph node or vascular organ like thyroid
- Clean glass slides: Frosted glass slides are preferable because they are easy to be labeled
- Spirit swabs: Clean sterile swabs soaked with spirit are needed to clean the area of aspiration
- Ninety-five percent ethanol for fixation of slide: This is needed to fix the slides for Papanicolaou's (Pap) stain
- Few capped vials containing 10% formalin solution for cell blocks. Cell block is needed to do immunocytochemistry on FNAC sample

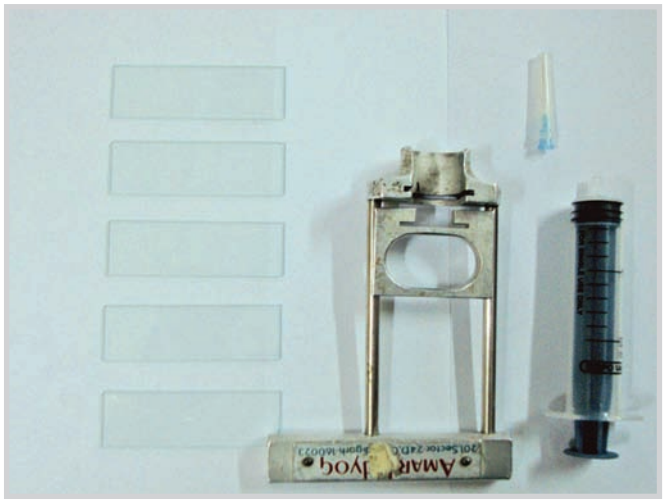

Fig. 15.1: Equipments needed for FNAC is highlighted here: glass slides, pistol handle, needle and syringe.

BOX 15.5 Fine needle aspiration procedure

- *Clinical history*: Always take clinical details before FNAC
- *Preparation of the patient*:
  - Explain the technique
  - Take proper consent
- *Aspiration*:
  - Clean the area by spirit swab
  - The swelling is hold in between the two fingers to make it immobilized
  - Needle is inserted within the mass
  - To and fro movement of the needle
  - Intermittent negative suction
  - Suction stopped
  - Needle is withdrawn
  - Needle is detached from the syringe
  - Syringe is filled with air
  - Material pushed on glass slide
  - Multiple smear made
  - Pressure by cotton swab is applied to the site of FNAC

- Few capped vials containing balanced salt solution for transport of the material in of case flow cytometry is needed.

FINE NEEDLE ASPIRATION TECHNIQUE

Clinical history: Before performing FNAC procedure, a good clinical history is mandatory. It gives the idea of the provisional diagnosis so that the operator manages to take samples for ancillary tests. Clinical history is very important for interpretation of FNAC smears.

Preparation of the patient: Many times, the patient is apprehensive and therefore a good talk with the patient makes him/her relax. I have noted that the patient often feels that the biopsy is a cumbersome process so they keep themselves fasting overnight. These patients are vulnerable to collapse or faint. It is the duty of the cytologist to take special care of such patients. All the patients should be explained about the whole procedure. Explanation of the technique makes them more understandable and cooperative. Depending on the situation and country's law, one should take proper consent from the patient for the FNAC procedure. This is particularly true for FNAC of deep seated lesions and orbit. The area of the site of FNAC should be properly cleaned with a spirit swab.

Aspiration: It is generally recommended that the cytopathologist himself/herself should perform FNAC.[7,8] The plastic syringe with attached needle is properly fitted to the pistol handle. The swelling is held in between the two fingers to make it immobilized. The needle is gently and swiftly introduced within the mass. The needle should be directed perpendicular of the swelling and should penetrate adequately. There should be to and from movement of the needle within the mass. In between, gentle negative suction should be given (**Box 15.5**). The needle may be withdrawn a little within the mass and the direction of the needle should be changed slightly. Major change of direction of the needle is not advisable as this may cut the tissue significantly. At the end of the FNAC, the needle is withdrawn sharply along with the release of the plunger to stop the suction. The majority of times the material remains in the needle hub. Therefore, releasing the negative pressure before withdrawing the needle is a vital step to get adequate material. The needle is then rapidly detached from the syringe and the plunger is retracted to get enough air within the syringe. The needle is reattached and the air is pushed to eject the material on the glass slide. Firm pressure should be applied on the site of FNAC to prevent any hematoma formation (**Figs 15.2** to **15.6**).

Smear Preparation

The material is pushed on the clean glass slide a little away from the corner. The direction of the needle should be along the long axis of the slide to avoid spillage of the material sideways. Now the smear is made by gently pressing a clean glass slide over it and moving the upper slide over the lower one to spread the material. Multiple smears are made and both air dried and alcohol-fixed smears should be kept for staining. The residual material should be rinsed in balanced salt solution and can be processed for ancillary techniques. If needed, a repeat FNAC should be undertaken to get more material for the ancillary techniques (**Figs 15.7** and **15.8**).

Fine needle sampling: Fine needle sampling (FNS) is an alternative technique of FNAC and is helpful in certain situations like thyroid swelling, breast lesions and small lymph nodes in a child.[9] In FNS procedure, no syringe or pistol handle is required during the procurement of the sample. The swelling should be pressed in between the two fingers and a thin bore needle is gently introduced. The needle is moved to and fro in the same direction and also in small inclination in different directions. The material

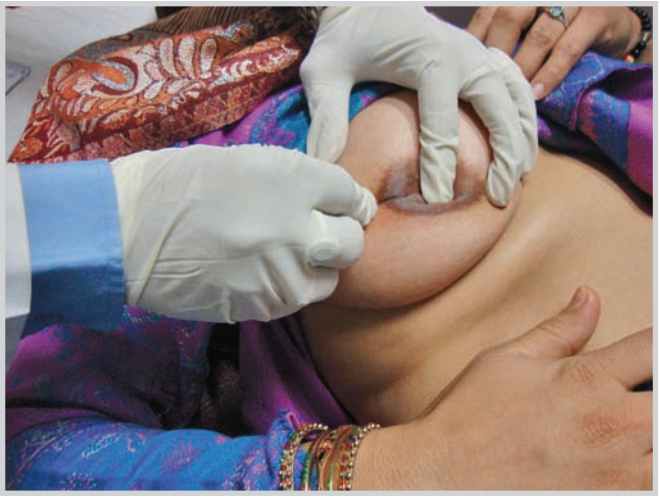

Fig. 15.2: Site of FNAC is cleaned by a clean spirit swab

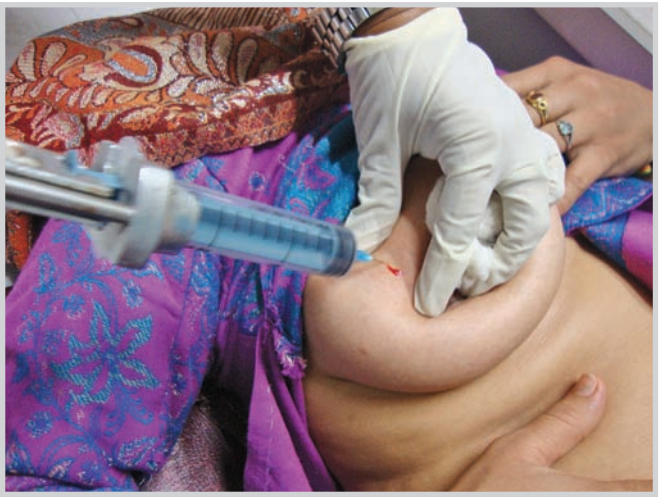

Fig. 15.3: The needle attached with the syringe is introduced into the swelling

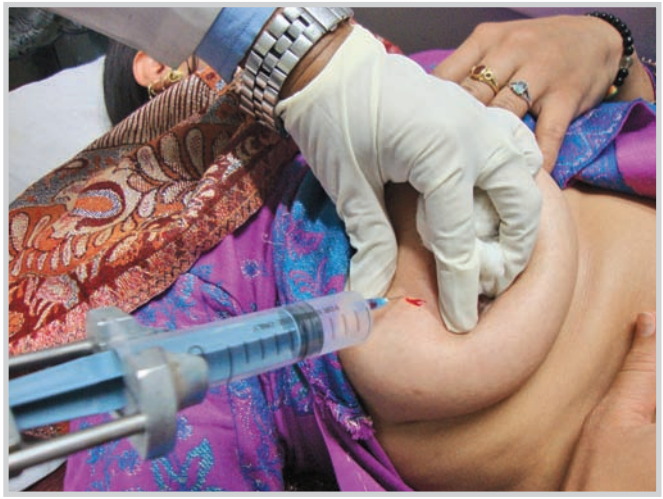

Fig. 15.4: The syringe and needle are moved to and fro and simultaneously negative suction is given

Fig. 15.5: The needle with the syringe is withdrawn from the swelling and the needle is detached from the syringe

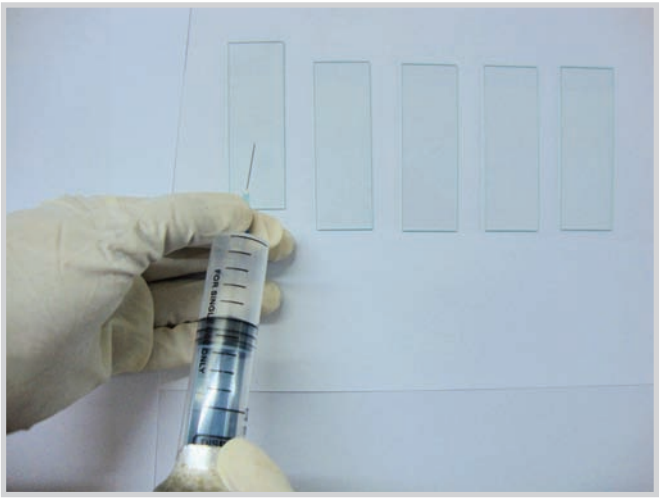

Fig. 15.6: Air is filled in the syringe, and the material is expelled from the syringe and needle hub to the glass slide

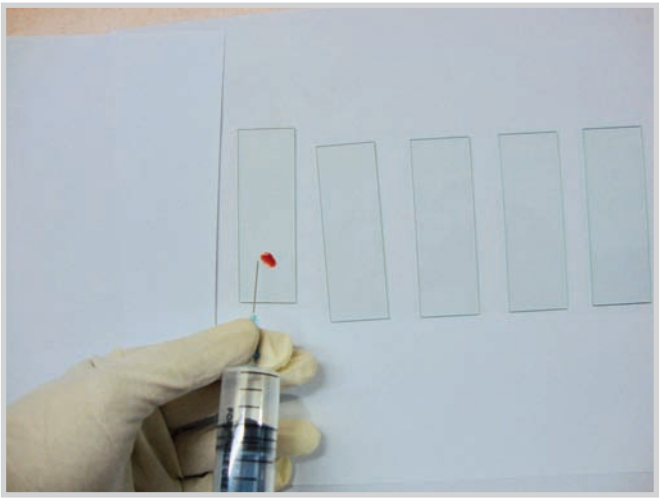

Fig. 15.7: Material is expelled on the glass slide

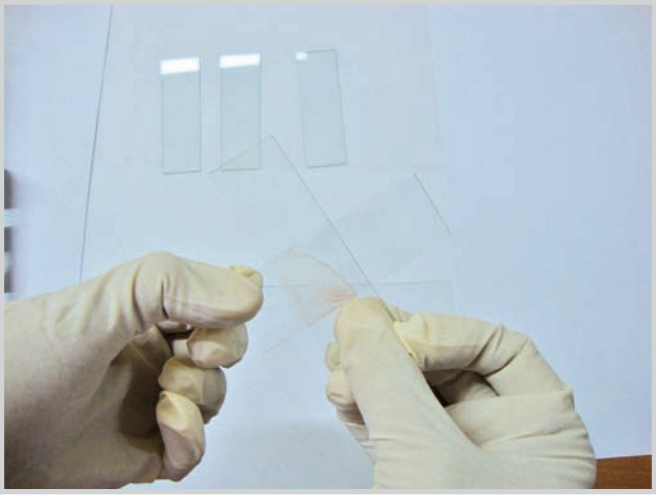

Fig. 15.8: Smear is made by putting another slide on the material and gently pushing the two slides apart

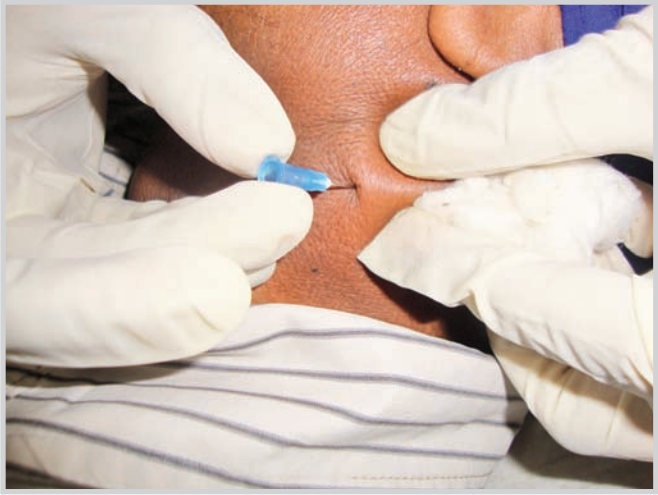

Fig. 15.10: The swelling is compressed in between the two fingers and the needle is introduced to the swelling gently

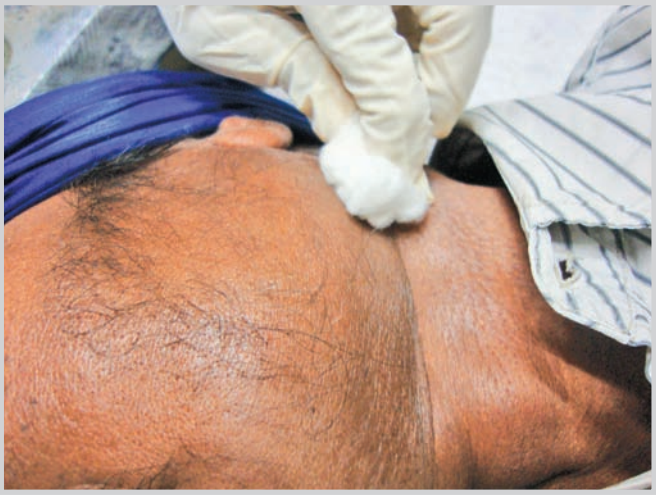

Fig. 15.9: Site of FNS is cleaned

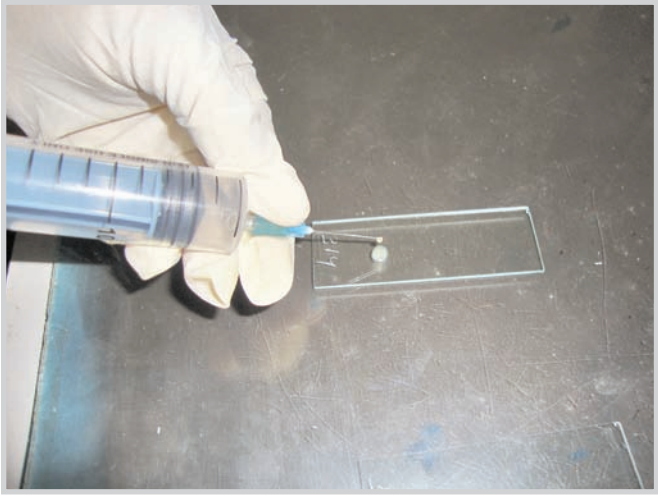

Fig. 15.11: The needle is withdrawn and the material is expelled on the glass slide

comes to the hub of the needle by capillary pressure. The needle is gently withdrawn and the syringe filled with air is attached to the needle hub. The aspirated material is expelled gently on the slide with the help of the syringe (**Figs 15.9** to **15.11**).

The major advantage of FNS is to get material without any admixture of blood (**Box 15.6**). Therefore, FNS is particularly important for vascular organs like thyroid. The needle also can be easily manipulated as it is free of any attached syringe. However, the material is usually less in volume and cannot be divided into many slides. Moreover, FNS is not suitable for cystic lesion as the fluid is spilled over in this technique. In hard and fibrotic lesion, this technique is unsuitable.

BOX 15.6 **Fine needle sampling technique**

Indications:
- Vascular tissue such as thyroid
- Small mobile lymph node
- Soft and mobile lesions in breast

Advantages:
- Least painful
- Easy to fix the lesion by needle
- Completely devoid of blood
- Good material

Limitations:
- Not good for cystic lesion due to spillage of fluid
- Material cannot be divided into many slides

Staining of the Smear

Fixation: Air-dried smears are used for May-Grünwald-Giemsaa (MGG) staining. For routine Pap staining 95% ethanol, methanol or isopropyl alcohol is used for fixation. The smears should be fixed at least 30 minutes. Commercially available spray fixatives can also be used as an alternate to wet fixation. The components of these spray fixatives are usually ethanol or isopropyl alcohol along with polyethylene glycol (carbowax). Within a few seconds, the smear is dried and carbowax provides a covering over the slide that prevents the shrinkage of the cell. Before staining, the

smear should be dipped in ethyl alcohol for at least 15 minutes to remove the surface coating. In case of rapid Pap stain, only air dried smears are used.[10]

Staining

Air dried smear: MGG, Romanowsky stain or Diff-Quik stain is done on the air dried smear. In Romanowsky stain, there is a mixture of methylene blue and Azure A, B or C. This is a metachromatic stain and thereby stains epithelial cells differently than stromal cells. This is also a good stain for mucin and extracellular matrix.

Wet fixed smear: On alcohol fixed smear, either Pap stain or hematoxylin and eosin stain is used. One great advantage of Pap stain is to demonstrate intracellular keratin and thereby identification of squamous cells.

Rapid staining: Commercially available Diff-Quick stain can be used for rapid staining within 2 minutes. Rapid Pap stain can also be done within 3 minutes.[10]

Ancillary Techniques

All the ancillary techniques used in tissue section can also be done in FNAC sample.[11] Depending on the resource and the situation, these tests should be done accordingly. However, most of the laboratories routinely do a cell block for immunocytochemistry and flow cytometry for immunophenotyping.

FNAC of Deep Seated Lesions

Fine needle aspiration cytology of the deep seated lesions in the body is almost similar to FNAC of superficial lesion. Radiological guidance is needed to perform FNAC of deeply seated lesions of the body. Certain information is needed before performing FNAC of radiological guided lesions (**Box 15.7**).

The deep seated lesion can be aspirated under the guidance of ultrasonography (USG), computerized tomography (CT) or fluoroscopy (**Figs 15.12** to **15.15**). Depending on individual situations radiologist decide the mode of guidance for FNAC of deeper lesions. USG guidance of FNAC is the most popular as it is easy to perform, less expensive and rapid technique (**Box 15.8**).

Computerized tomography guided FNAC is more appropriate for small lesion which needs precise localization (**Box 15.9**). However, this is a time consuming and expensive procedure.

The longer needle is used for USG and CT guided FNAC. Local anesthesia is needed for radiological guided FNAC. The rest of the procedure is same as that of superficial FNAC. It is preferable to have immediate rapid Giemsa stain for assessment of the aspirated material.[12]

BOX 15.7 **Prior information needed for guided FNAC**

- Clinical history
- Location of the lesion
- Size of the lesion
- Consistency of the lesion
- Routine blood test
- Coagulation parameters

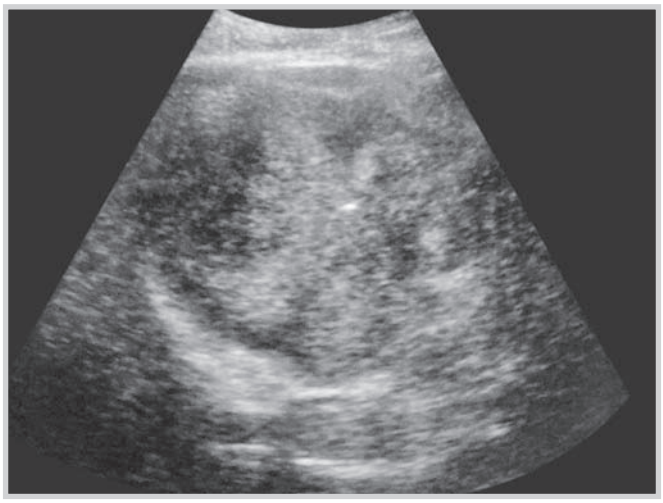

Fig. 15.12: Ultrasound guided FNAC of the space occupying lesion of the liver (*Courtesy:* Dr Anupam Lal, Additional Professor, PGIMER, Chandigarh, India)

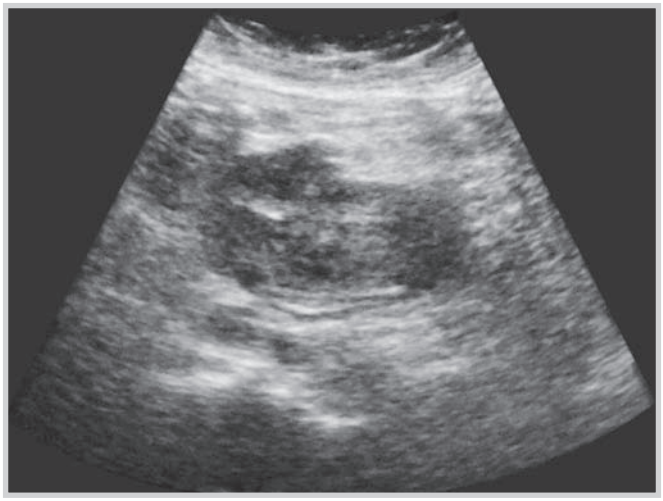

Fig. 15.13: Ultrasound guided FNAC of mass in the pancreatic head (*Courtesy:* Dr Anupam Lal, Additional Professor, PGIMER, Chandigarh, India)

Transrectal FNAC of the Prostate

Fine needle aspiration cytology is widely used for transrectal FNAC of the prostate. This procedure is easy to perform and multiple sampling can be done from the different areas of the prostate.

Franzen's guide and needle are needed for doing transrectal FNAC of the prostate. Franzen's guide is the long guide with a central hole inside (**Fig. 15.16**). Franzen's needle is introduced within the guide hole for FNAC of the prostate (**Fig. 15.17**).

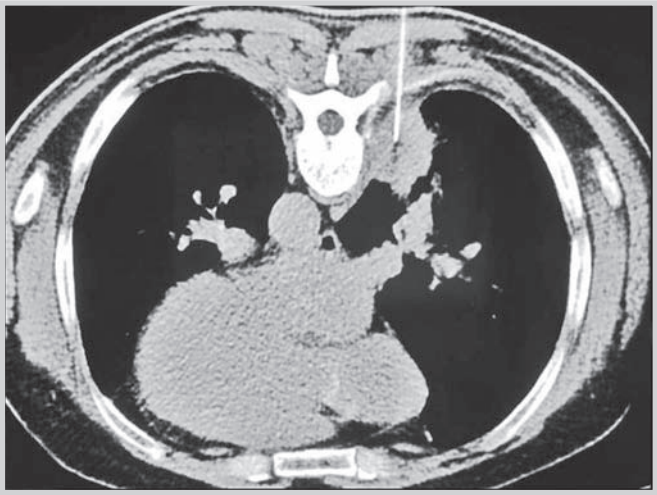

Fig. 15.14: Computerized tomography guided FNAC of a peripheral lung mass (*Courtesy:* Dr Anupam Lal, Additional Professor, PGIMER, Chandigarh, India)

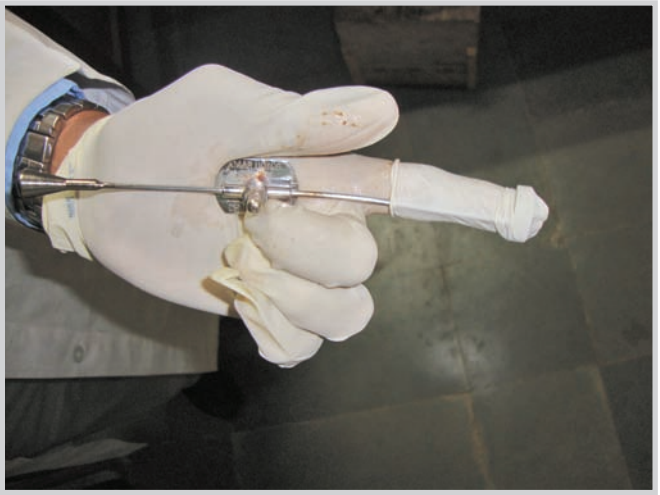

Fig. 15.16: Franzen's guide fixed with the finger

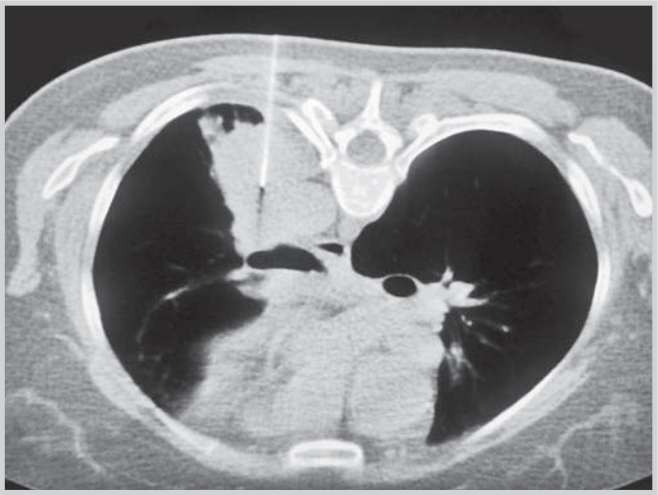

Fig. 15.15: Computerized tomography guided FNAC of lung mass abutting the aorta (*Courtesy:* Dr Anupam Lal, Additional Professor, PGIMER, Chandigarh, India)

Fig. 15.17: Franzen's needle is introduced within the hole of the guide for FNAC of the prostate

BOX 15.8 Ultrasonography guided FNAC

- Easy to do
- Cheap
- Rapid
- Position of the patient can be changed
- Real time
- No radiation exposure
- Significant obscuration due to air or bone

BOX 15.9 Computerized tomography guided FNAC

- High resolution
- Exact localization of the needle
- Image of the traversing needle visible
- Costly
- Time taking procedure
- Radiation exposure

At first, the patient should lie down in his left lateral position with the lower leg extended and upper leg folded to chest firmly. The prostate should be palpated to assess initially. Franzen's guide should be fixed with the left middle finger of the aspirator with the help of finger stall. Now the Franzen's guide is introduced gently through the rectum to the area of the prostate to be aspirated (**Fig. 15.18**). FNAC is now done with the help of Franzen's needle within the guide and syringe (**Fig. 15.19**).

BOX 15.10 Nonrepresentative material

- Excessive blood
- Insufficient material
  - Wrong technique of FNAC
  - Scarred tissue
- Crushing artifact
- Air drying artifact
- Thick smear

BOX 15.11 Checklist for evaluation of the FNAC smear

Clinical:
- Chief complaints of the patient
- Sex and age
- Any pertinent sign
- Location of the lesion
- Radiological or ultrasound features
- Laboratory data
- Gross appearance of the aspirated material

Cytological data:
- Cellularity
- Cell arrangement or distribution
- Cell population
- Cell morphology
- Background noncellular materials

Suboptimal Material

The FNAC material may be suboptimal for various reasons. The excessive blood in the smear may be present due to too much suction in a vascular lesion. This can be avoided by intermittent suction when applying negative suction. Alternatively, FNS can grossly reduce the amount of blood in the sample. Insufficient material may be due to the wrong technique of FNAC. The needle may not hit the mass properly or the movement of the needle may not be sufficient to dissociate the tissue. At times, the mass may be too much sclerotic or hard and therefore, even after adequate negative suction, the material may not be obtained. The other causes of suboptimal smear for interpretation are air dried smear and thick smear (**Box 15.10**).

Evaluation of FNAC Smear

An FNAC smear of each case should always be evaluated along with the clinical history. The clinician should always give feedback to the cytologist. For medicolegal issues and to avoid false positive cases, a repeat FNAC should be done or if necessary, tissue biopsy should be advised. For the initial evaluation of FNAC smear, the cytopathologist should always have the checklist of the important features as mentioned in the **Box 15.11**.

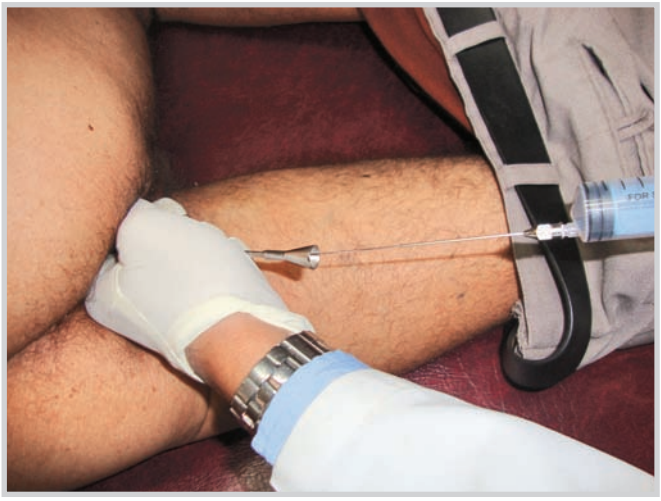

Fig. 15.18: Franzen's guide along with the needle is introduced gently through the rectum

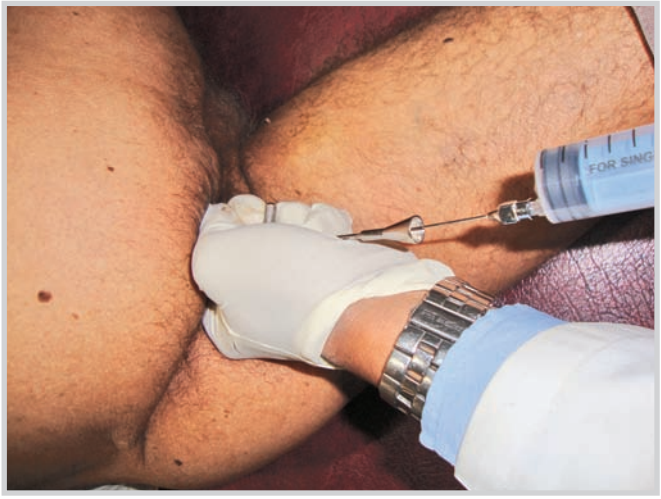

Fig. 15.19: The needle traverses through the glove. It is moved to and fro, and negative suction is applied by syringe

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CHAPTER 16

Routine Laboratory Techniques

Chapter Contents

INTRODUCTION

In the last few decades, there is a massive advancement in laboratory techniques due to the introduction of various newer technologies. In spite of the modern technologies, the simple routine laboratory techniques are very important because basic staining of the smear and its morphological interpretation are essential for diagnosis. The optimum function of a laboratory depends on the proper collection of sample, fixation of the material for cellular details, processing, staining and accurate interpretation of the sample.

SAMPLE COLLECTION

Cervical Cytology

Preparation of the patient: Proper collection of cervical cytology sample has immense importance to avoid false negative rate. For the optimum collection of cervical smear, the patient should take an appointment preferably two weeks after the first day of the last menstrual period. The patient should not use vaginal cream, jellies or tampons at least two days prior to the test. She also should be refrained from sexual intercourse 48 hours before the test.

Labeling the sample: The glass slide or the vial [(for liquid- based cytology (LBC)] must be labeled by unique identifier to prevent any mix up. The marker on the slide should be permanent so that it is not removed during processing of the smear. As frosted sample is costly, so in our institution we use a diamond pencil to write the number on the slide.

Collection devices: To date, no collection device has been universally accepted to get adequate Pap smear in all patients. The main target area of any collection device is the transformation zone. This is a dynamic area and may move upwards or downwards depending on the patient's age, parity, menstrual cycle, inflammation or premalignant condition of the cervix. The main aim of the cervical device is to collect the material from this transformation zone. The ideal sampling device should have certain advantages such as it should be accessible to both ectocervical and endocervical regions, the material should not stick to the device, it should not traumatize the region too much and the device should be cheap. Various types of cervical spatula and brushes are now available as a collection device (**Fig. 16.1**).

- Wooden spatula
- Plastic spatula
- Endocervical brush
- Cervex-Brush®

Each device has certain advantages and disadvantages[1] (**Table 16.1**). Wooden spatulas are available in disposable form. They are cheap , easy to use and relatively atraumatic. However, the cells may be trapped in the wood and endocervical cells are less in amount in wooden spatula. Plastic spatulas are a bit costlier than wooden spatulas. However, they prevent cells trapping. The cotton tip applicators are minimally traumatic, cheap and give minimal discomfort to the patients. However, the cells may be trapped in the cotton fibers. Endocervical brush is

TABLE 16.1: Advantages and disadvantages of different collection devices

Collection device	*Advantages*	*Disadvantages*
Wooden spatula	• Cheap • Easy to use • Relatively atraumatic • Minimal blood contamination	• Cells may be trapped into wood • Endocervical cells are less
Plastic spatula	• Plastic prevents the cells to get trapped	• Costly than wooden spatula
Endocervical brush	• Easy to insert into the endocervical canal • Minimal entrapment of the cells with the brush	• Trauma to the endocervix by stiff bristles so bloody smear • Painful • Chances of air drying
Cotton tip applicator	• Minimum discomfort • Low cost • No trauma • Good amount of endocervical cells	• No ectocervical cells • Cells may be trapped into cotton fibres
Cervex-Brush®	• Samples both ecto and Endocervix on one slide	• Costly

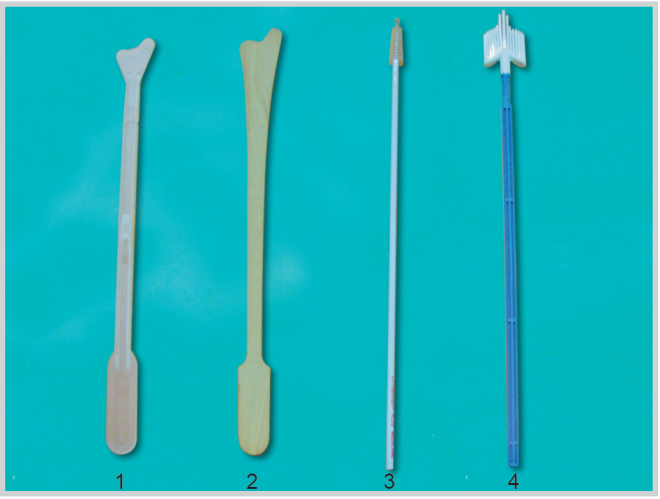

Fig. 16.1: Different sampling devices for the collection of cervical smear: (1) Plastic spatula, (2) wooden spatula, (3) endocervical brush, 4) Cervex-Brush®

easy to introduce into the endocervical canal and it provides a good amount of endocervical cells. There is minimal entrapment of the cells by this brush. However, the stiff bristles may cause damage of the cervical tissue. The Cervex-Brush® provides both ecto and endocervix on one slide. It is one of the most suitable devices. However, these are costlier than any other sample collection devices.

In a meta-analysis study, it has been suggested that the most effective collection device appears to be a combination of the cytobrush with an extended tip spatula.[2]

Collection Proper

The position of the patient: The cervical smear is taken by keeping the patient in dorsolithotomy position. In this position, cervix is mostly visualized (**Box 16.1**).

BOX 16.1 Cervical smear collection

Preparation of the patient
- Do not use vaginal cream, jellies or tampons at least two days prior to the test
- Should not be in menstruation period
- No sexual intercourse within 48 hours

Collection devices
- Cervical brush
- Endocervical broom stick
- Plastic spatula
- Wooden spatula
- Collection proper
- Position of the patient: Dorsolithotomy
- Preparation
  - The vagina is cleaned by wet swab with water
  - No lubricant jellies should be used
  - The plastic spatula is inserted within in the vagina
  - The spatula is rotated 360°
  - The sample on the spatula is spread on the glass slide
  - The slide is immediately immersed in 95% ethanol for fixation

Preparation: The vagina is cleaned by wet swab. Water may be used for lubricant, however, no lubricant jellies should be used. A speculum is introduced to visualize the cervix. The main focus should be on the transformation zone as most of the preneoplastic lesions develop from this area. Proper inspection of the ectocervix and transformation zone is required and smear should be taken from the abnormal area first. Ectocervix is smooth pink and opaque whereas the endocervix is dark pink.

Collection: Plastic spatula and endocervical brush are optimum to take smear. At first, the plastic spatula is inserted within in the vagina and the tip of the spatula fits with the contour of the cervix. The spatula is rotated 360° and then the spatula is withdrawn with the material on the one surface. Next endocervical brush is introduced and the tip of the brush should fit within the endocervical canal.

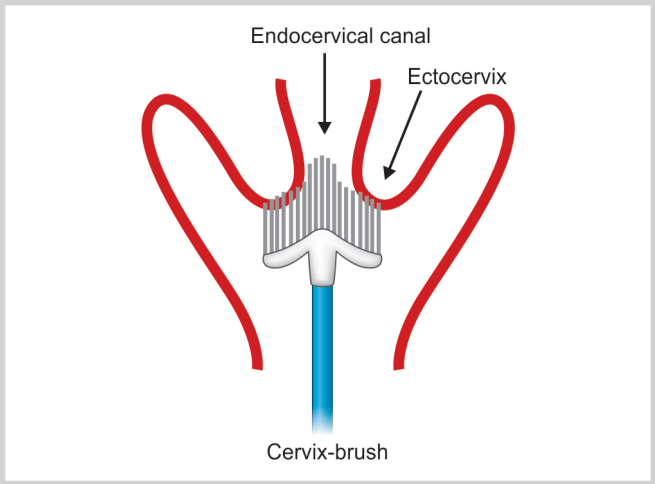

Fig. 16.2: Method of collection of cervical smear with the help of Cervex-Brush®

The brush is gently withdrawn. The sample on the spatula is first spread on the one half of the glass slide by longitudinal stroke followed by rolling of the endocervical brush on the other half of the glass slide. The Cervex-Brush should fit with the endocervical canal. The bristles are fitted with the ectocervix and the tip of the brush is within the endocervical canal. The brush is rotated 360° and the material is obtained (**Fig. 16.2**).

In case of LBC, the samples from both spatula and endocervical brush is rinsed into the collection fluid supplied by the manufacturers and then the spatula and brush are discarded.

Vaginal smear: The speculum is gently introduced and the lateral vaginal wall is visualized. With the help of a spatula, scraping is done from the lateral vaginal wall and the smear is prepared.

Endometrial aspiration smear: A sterile canula is inserted into the uterine cavity and with the help of a syringe the material is gently sucked. The aspirated material is spread onto the glass slide and fixed immediately.

Sputum

A morning specimen of sputum should be obtained in a clean wide mouthed container. If necessary, aerosol can be used to get the sputum. Sputum should be sent fresh to the laboratory and no fixative is recommended.

Fluid

Body cavity fluids are collected in a dry clean container as a fresh sample.[3,4] It is preferable to collect the fluid without any fixative and the material should be sent as fresh sample to the laboratory for immediate processing. In our laboratory, we collect the fluid in anticoagulant solution to prevent clotting of the fluid. We use 1:9 ratio of ammonium oxalate:fluid. The collected fluid may be kept in a refrigerator if the processing of the fluid is not possible immediately. If a long delay in processing is expected, then an equal amount of 50% ethyl alcohol is mixed with the fluid during the time of collection of the sample.[3] Under no circumstances, fluid for cytological examination is allowed to be frozen.

Urine

The urine sample should be collected 3–4 hours after last micturation. Fresh randomly voided urine specimen should be collected in a clean container and should be sent to the laboratory for immediate processing to avoid any degenerative changes.

WASHINGS

Washing samples are usually sent as fresh without any fixative. The sample should be processed immediately. If a delay in the processing is expected then the fluid is mixed with equal amount of 50% ethanol.

BRUSHING

Brushing can be submitted in three ways[4]

1. Direct smear
2. Contents of the brush
3. Tip of the brush

Direct smear: The brush is gently rolled over a clean glass slide within one inch area. The slide is then immediately fixed by immersing in 95% ethyl alcohol or by spray fixative.

Brush contents and brush: Brush is dipped into the container with a physiologic transport solution and is shaken vigorously. Equal volume of 50% ethyl alcohol is mixed and then the whole container is sent to the laboratory. Alternatively, the tip of the brush can be dipped and cut in the liquid supplied by the manufacturer of the liquid based system. The whole thing is sent to the laboratory. Separate set of fixative should be used for each brush to prevent any contamination.

FIXATION

Preservation of the cellular detail is necessary for studying the cell and to make a diagnosis. It is very much essential to prevent autolysis and to keep the cell as close as possible to the living stage in the body. Fixative prevents the autolytic degradation of the cell by promoting rapid coagulation of the various enzyme proteins. The cytological fixative should have some essential properties such as rapid action, prevention of cellular distortion, facilitation of staining and preservation of nuclear detail (**Box 16.2**).

Wet Fixatives

For Papanicolaou's stain and Hematoxylin and eosin stain various fixatives are used.

1. *95% ethyl alcohol:* It is the most commonly used fixative. Ethanol causes dehydration of the cell and mild shrinkage. Before doing Papanicolaou's stain, hydration is necessary in the alcohol fixative smear.
2. *100% methanol:* It is equally effective as 95% ethanol. However, it is not cost effective.
3. *Denatured alcohol:* This is also equally good. The main advantage of denatured alcohol is that it is unsuitable for human consumption.

Time of fixation: The smear should be kept in this fixative for at least 30 minutes. Prolonged fixation usually does not alter the

BOX 16.2 Fixation

Desirable properties of fixatives
- Rapid action
- Prevention of cellular distortion
- Maintaining nuclear details
- Facilitation of staining
- Inactivation of microbial organisms
- Fixing the cell on glass slide

Routine fixatives for Papanicolaou and Hematoxylin stains
- 95% ethyl alcohol
- 100% metahnol
- Denatured alcohol

Time of fixation: 30 minutes
Hemorrhagic fluid: Carnoy's fixative
Fixatives for Liquid based preparation:
Thin prep system: Methanol based preservative
Surepath system: Ethanol based preservative
Cell Block: 10% neutral buffered formalin
Immunocytochemistry: 95% Ethanol
Electron microscopy: Glutaraldehyde solution (2.5%)

morphology of the cells. However, it is better to keep the slides in the refrigerator in a closed bottle or jar.

Coating Fixatives

Different varieties of spray fixative are available in the market from different companies. The fixative may be applied as spray or drops to coat the smear (**Box 16.3**). The main advantage of spray fixative is to avoid bottles with liquid fixative. There are two major functions of spray fixatives: (1) Cell fixation and (2) Protective covering of thin wax over the smear. The major ingredients of coating fixatives are alcohol and wax. So before staining procedure, the wax should be removed by keeping the smear in 95% ethanol.

The coat fixatives are particularly used for cervical smears. However, the coating fixatives are not recommended for smears prepared from fluid specimen. It is also not recommended for bloody smears as spray fixatives may cause RBC clumping. The spray fixative should be applied from optimal distance which is considered as 10–12 inches away from the smear.

Special Fixatives

Hemorrhagic Fluid

Carnoy's fixative: It is made of 95% ethanol (60 mL), chloroform (30 mL) and glacial acetic acid (10 mL). This fixative is the most suitable for blood-mixed smear as acetic acid lyses the blood. The nuclear detail of the cell is well preserved. However, Carnoy's fixative may cause remarkable cell shrinkage and over staining of nucleus by hematoxylin. This fixative should always be used fresh and should be discarded after use.

BOX 16.3 Spray fixatives

Advantages
- To avoid bottles with liquid fixative

Functions
- Cell fixation
- Protective covering of thin wax over the smear

Main use
- Cervical smear

Not Recommended
- Smears prepared from fluid specimen
- Bloody smears

Optimal distance of spray
- 10–12 inches away from the smear

Fixatives for Liquid-based Preparation

Presently, two types of liquid-based systems are available in the market: ThinPrep and SurePath. The ThinPrep system supplies methanol based preservative and SurePath supply ethanol based preservative for fixation of the cervical samples.

Cell Block

Ten percent neutral buffered formalin: Neutral buffered formaldehyde solution contains 40% formaldehyde (100 mL), water (900 mL), acid sodium phosphate (4 gm) and anhydrous disodium phosphate (6.5 gm). This is the most suitable solution for cell button fixation to make a cell block.

Immunocytochemistry

Ninty five percent ethanol: Routine fixative such as 95% ethanol can be used for immunocytochemistry.

Electron Microscopy

Glutaraldehyde solution (2.5%) is most suitable for electron microscopy as it fixes the cell rapidly and preserves the cellular organelles to study.

PRESERVATION OF SAMPLE PRIOR TO PROCESSING

It is expected that the procured sample should be processed immediately. However, it may not always be feasible to process the sample immediately. Specimen with high mucus and protein content can be kept for a few hours to days before processing (**Box 16.4**). Specimens with no sugar or protein, with extreme pH cannot be kept for a long time.

BOX 16.4 **Preservation of sample prior to processing**

Specimen with high mucus content: 24 hours to keep in refrigerator
- Sputum

Specimen with high protein content: 24–48 hours to keep in refrigerator
- Peritoneal, pericardial and pleural fluid

Specimen with low mucus and protein content: 1–2 hours
- Urine
- CSF

Specimen with low pH: Immediate processing
- Gastric aspirate

PROCESSING OF LABORATORY SAMPLES

Processing of a laboratory sample includes several steps of receiving, preparing smear, staining, to final submission of the slide for interpretation.

Receiving the Sample

Requisition Form

The sample should always be accompanied with a proper requisition form (**Box 16.5**).

Glass Slides and Liquid

The slide should be properly fixed and labeled. It is important to keep the slide in proper container to prevent the break of the glass slide. The slide should not be wrapped by requisition form. The paper form should be sent separately. Similarly, liquid sample should always be in an air tight container with proper labeling (**Box 16.6**).

Unique Identification Number

It is preferable to check the unique identification number of the sample and requisition form before processing the sample.

Laboratory Bar Code

A unique laboratory bar code number can be generated by the computer for each sample and this can be put on the container and smears and forms.

Processing

Processing of different sample is highlighted in **Table 16.2**.

BOX 16.5 **Requisition form**

- Name
- Age
- Sex
- Unique identification of the patient (hospital entry number)
- Date of collection
- Site and procedure of collection
- Clinician's name, telephone or fax number
- Tests to be done

Clinical history
- Chief complaints
- Salient physical findings
- Radiological features
- Important history: surgery, chemotherapy, radiotherapy, exposure of chemicals, history of immunosuppressions, etc.

BOX 16.6 **Receiving the samples**

Slide
- Slide should be sent in a shock resistant container
- Slide should be properly labeled
- Paper form should be in a separate bag

Liquid
- Air tight container
- Proper labeling of the container
- Plastic container is preferable than glass container in case of large amount of liquid sample

TABLE 16.2: Processing of sample

Sample	*Processing*
Moderate amount of fluid (40–50 mL effusion fluid, 2–5 mL washing etc.)	Centrifuge
Large amount (100 ml to 1 l) of clear fluid (urine)	Millipore filtration
Small amount of fluid (less than 1 ml such as CSF, vitreous fluid, ureteric sample, etc.)	Cytocentrifuge
Sputum	Pick and process method
Material for immunocytochemistry	Cell block preparation

Processing of Sputum

Sputum should be kept in a PetriDish and carefully examined on a black background. Any tissue fragments or gray white substance or bloody material should be picked up by a clean forceps and smears are prepared on the clean glass slide.

Processing of Fluid

Centrifuge: Fluid specimen should be put in clean air tight centrifuged tube. It is preferable to use a plastic tube than glass tube. The sample is then rotated at 1500 rounds per minute (RPM) for 10 minutes. The supernatant liquid is discarded and multiple smears are made from the sediments.

Cytocentrifuge: If the quantity of the sample is small then cytocentrifugation is done in 1000 RPM for 5 minutes. A thin layer of smear is formed on the glass slide. The smears are immediately fixed in 95% ethanol for staining.

Centrifugation: A centrifuge is one of the important separation devices by which the particles in a solution are separated according to their size, shape, density and viscosity of the suspension medium (**Figs 16.3** and **16.4**).

Basic principle: In a liquid suspension, the particles whose density is higher than the liquid sink and the lighter particles float. The movement of the particles in the liquid media greatly depends on the density difference with the suspension liquid. Centrifugation technique replaces the gravity by a more powerful "centrifugal force" to enhance the rate of sedimentation of suspended particles that are denser than the suspension liquid (**Fig. 16.5**). This effective gravity increases depending on the radius of the arm of the centrifuge machine and the square of the rotational rate of the arms.

Relative centrifugal force = mass × radius of rotation (that means, distance of particles from the axis) × (angular movement)^2

The principle of cytocentrifuge is similar to that of the centrifuge. There are two main advantages of cytocentrifuge: (1) small volume of fluid is processed, and 2) the smear is directly prepared on the glass slide.

There are two types of commercially available cytocentrifuge: (1) the cytocentrifuge that removes the fluid during the time of sedimentation (**Figs 16.6** to **16.8**) and (2) the cytocentrifuge that retains the fluid. When the fluid is removed during the time of sedimentation, the cells experience hydraulic force due to the flow of fluid away from the sample area. The sedimentation velocity should be greater than hydraulic force and a high cell recovery depends on the balance between the centrifugal force and hydraulic force during the machine operation time. In another type of centrifugation machine, where the fluid is not removed during sedimentation, the centrifugal force

Fig. 16.3: Centrifuge machine

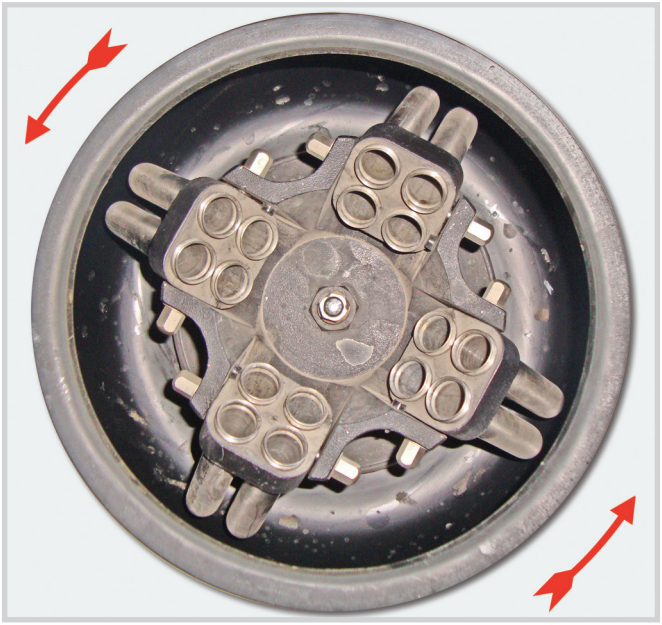

Fig. 16.4: Interior of the centrifuge machine

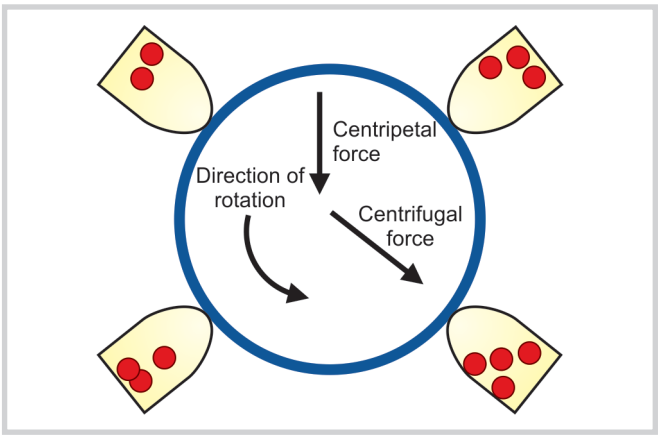

Fig. 16.5: Basic principle of centrifugation is highlighted by this schematic diagram. Centrifugation technique replaces the gravity by a more powerful "centrifugal force" to enhance the rate of sedimentation of suspended particles that are denser than the suspension liquid

Fig. 16.6: Cytocentrifuge machine

Fig. 16.7: Interior chamber of the cytocentrifuge machine

should be adequate to flatten the cells on the glass slide. The supernatant fluid should also be removed carefully. Cytocentrifugation may cause morphological distortion of the cells and careful attention should be given in this aspect.

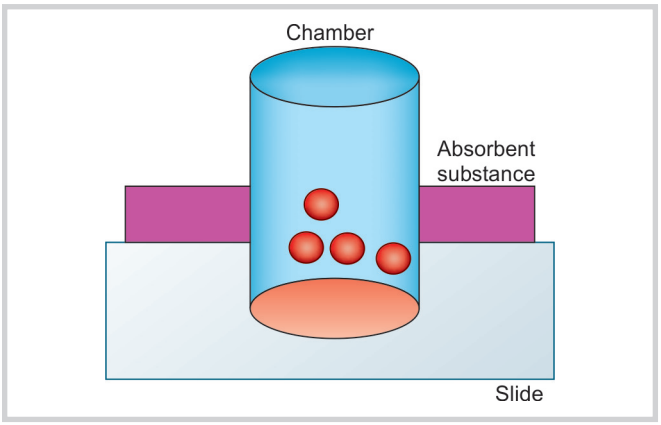

Fig. 16.8: Basic process of the cytocentrifuge technique. Here the liquid suspension medium is absorbed by the absorbent

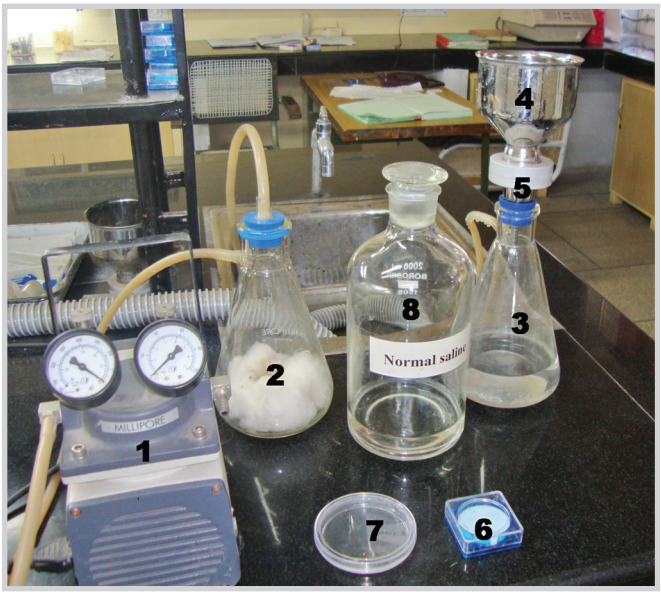

Fig. 16.9: Basic equipments of the Millipore filtration technique are shown: (1) Pump for negative suction, (2) Conical flask, (3) Conical flask attached with filter cup, (4) Filter cup, (5) Sieve, (6) Millipore filter paper, (7) Petri Dish, (8) Normal saline to moisten the filter paper

The optimum rotational speed of the machine is the most important factor in this respect.

Millipore Filtration

The Millipore filtration technique is done for processing large quantity of clear urine sample. The Millipore filter paper is moistened with normal saline and the paper is put on the sieve. The filter cup is attached and the sample is put in the filter cup. The negative pressure at 100 mm Mercury is created and the fluid is drained into the bottle leaving the cell on the filter paper (**Fig. 16.9**). Multiple imprint smears on albumin coated slides are made and the slides are fixed immediately in 95% alcohol for 30 minutes.

Processing of Hemorrhagic Fluid

Carony's fixative can be used for processing of hemorrhagic fluid.

Buffy coat preparation: This is an alternative way to process bloody samples. The hemorrhagic sample first centrifuged at 2000 RPM for 10 minutes. The supernatant fluid is discarded. The residual suspension is taken in 2–3 inches long capillary tube. The one end of the tube is sealed and then the capillary tube is centrifuged. The capillary is broken in the buffy coat region and fish tail preparation of smear is made.

Cell Block

The cell block technique helps to get the paraffin embedded tissue and thus multiple sections can be obtained to do routine hematoxylin and eosin stain, cytochemistry and immunocytochemistry. Another added advantage of cell block is to keep material for archival use. The cell block is made by the following steps:

1. The specimen is collected in 10% neutral buffered formalin
2. The specimen is kept 4 hours in formalin to fix the cells
3. Centrifuge the sample 1500 RPM for 10 minutes
4. Wash the sediment twice in Phosphate buffer solution (PBS) by centrifugation
5. Add 100 microliter of plasma and 30 microliter thrombin
6. The clot is removed and collected on filter paper
7. The clot is processed in the tissue processor.

Compact Cell Block Technique

Young et al.[5] described compact cell block technique that is in a small area and completely free of RBCs.

Steps:

1. Fluid containing the material is centrifuged.
2. CytoRich solution (designed for haemolysis and gentle fixation of the cell) is added in 1:1 ratio to the sediment and is kept for 2 minutes
3. Then 4 drops of plasma and three drops of thrombin (5000IU/mL) is added
4. The mixture is gently agitated to make a cell clot
5. The clot is transferred into the lens paper
6. The compact clot is wrapped and then fixed into formalin
7. The tissue is processed in the tissue processor.

Staining

Papanicolaou's staining is a worldwide accepted staining method and the staining technique is named as Dr George Papanicolaou, the father of Cytopathology. This stain is done in all cervical smear and non-gynecologic exfoliative smears. It is a multichromatic staining which helps in the display of cellular maturation, cytoplasmic details and nuclear details. It is a transparent stain so cells with overlapping nuclei can also be studied by this stain. Over the years, various modifications have been done on Papanicolaou's staining in laboratory to laboratory. Presently automated staining technique is available in many laboratories (**Fig. 16.10**).

Fig. 16.10: Automatic stainer

Progressive method: Here, the intensity of the nuclear stain is done up to the desired level and the cytoplasm barely takes the dye.

Regressive method: Here, the nuclei are deliberately over stained by haematoxylin dye and then excess stain is removed by diluted hydrochloric acid solution.

Dyes Used in Papanicolaou's Staining

Hematoxylin: Harris hematoxylin is used for nuclear stain

Orange G: OG-6 is used for cytoplasmic counter stain. This dye specifically stains keratin component of the cytoplasm.

Eosin Azure (EA): It is a polychrome stain and consists of three dyes: Eosin Y, light green SF yellowish and Bismarck brown Y.

Principle of Basic Steps

1. *Rehydration of the smear:* By gradual dip of the smear in graded concentration of alcohol.
2. *Nuclear staining by hematoxylin:* Harris hematoxylin is used for the nuclear stain. The excess hematoxyline is removed by 0.05% aqueous solution of hydrochloric acid. To make the stain more stable, the smear is further treated with a weak alkaline solution.
3. *Cytoplasmic staining by Orange G (OG):* The smear is again brought into alcohol and stained with OG. OG is an acidic dye and stains keratin as orange color.
4. *Cytoplasmic staining by EA:* EA is a synthetic dye and stains cytoplasm as blue green color.
5. *Dehydration by absolute alcohol*: Rinsing the smear with absolute alcohol causes dehydration.
6. *Clearing by xylene:* The smear is dipped in xylene. Xylene has the same refractive index as that of glass and mounting medium.
7. *Mounting by DPX:* Mounting medium should be miscible with the clearing agent. It prevents fading of the stain.

Papanicolaou's Staining Steps

1. 70% Ethanol: 1 minutes
2. 50% Ethanol: 1 minutes
3. Distilled water: 5 dips
4. Harris hematoxylin: three and half minutes
5. Distilled water: 5 dips
6. 0.5% aqueous solution of hydrochloric acid: few dips
7. Water: 1 minute
8. Lithium carbonate: one and half minute
9. Water: few dips
10. 70% Ethanol: 2 minutes
11. 90% Ethanol: 2 minutes
12. Orange G : few dips
13. 95% Ethanol: 2 minutes
14. EA modified: 2 minutes
15. Absolute ethyl alcohol : 2 minutes
16. Absolute ethyl alcohol : 2 minutes
17. Absolute ethyl alcohol and: 2 minutes
18. Xylene: 5 minutes
19. Xylene: till clear
20. Mounting in DPX

Hematoxylin Solution for Papanicolaou's Stain

Harris Hematoxylin: 10 gm

Water: 2 liter

Alum: 200 gm

Absolute alcohol: 100 mL

Mercuric oxide: 5 gm

EA Solution

Eosin: 4 gm

Light green: 0.4 gm

Water: 480 mL

Alcohol: 500 mL

Phospho tungstic acid: 2 gm

Glacial acetic acid: 20 mL

Precautions to be Taken in Papanicolaou's Staining

The following precautions should be taken in Papanicolaou's staining:

Staining Solutions

- The concentration of the stain should be checked regularly. The containers should always be covered to prevent evaporation of fluid. The stain solutions should be periodically replaced to maintain the quality of the stain. Hematoxylin should be changed every day and OG-6 and EA should be changed weekly. Xylene should be changed immediately if it is tinted.
- The stain solution should be free of stain deposits and should be filtered daily.

Containers

- The containers should be washed regularly.

Coverslip

- Smears should not dry before placing the coverslip.
- No air bubbles should be present between the coverslip and smear.

Staining Proper

- Minor agitation of the slides prevents staining deposit and gives better staining.

Too Dark Nuclear Stain

- If the smear is kept in Harris hematoxylin for a long time.
- Too less hydrochloric acid concentration
- Too less number of dips in hydrochloric acid

Too Pale Nuclear Stain

- If hematoxylin is diluted
- Too less bluing
- Too much concentration of hydrochloric acid
- Too much dip in hydrochloric acid
- Air-dried smear prior to fixation

Improper Cytoplasmic Stain

- Smear is air-dried prior to fixation
- If the slides remain in alcohol for too long time then the cytoplasmic stain may be pale.
- If the pH of EA is not optimum.
- If the slide remains in hematoxylin for too long time.

De-staining and Re-staining of the Smear

For de-staining of the smear the following steps are done in our laboratory in Post Graduate Institute of Medical Education and Research, Chandigarh:

1. The smear is kept in xylene until the coverslip drops.
2. The smear is the kept in acid alcohol for 20 minutes (80 mL of 95% ethanol and 20 mL of 0.5% hydrochloric acid).
3. The smear is then washed with running water.
4. The smear is re-stained.

Ultrafast Papanicolaou's Staining

Ultrafast Papanicolaou's staining was first developed by Young et al. for rapid staining of fine needle aspiration cytology. In this technique, the air-dried smear is first dipped in normal saline for 30 seconds. The smear is then dipped in alcoholic formalin for 10 seconds. After this, the smear is processed as usual for Papanicolaou's stain.[6]

May-Grünwald-Giemsa Stain

May-Grünwald-Giemsa (MGG) is a Romanowski's stain and is used in addition to Papanicolaou's and hematoxylin and eosin stain in many laboratories. This stain provides excellent

cytoplasmic details character. This is a metachromatic stain. MGG is a convenient stain for FNAC smear. **Table 16.3** compares the relative advantages and disadvantages of MGG stain over Papanicolaou's stain.

Steps

1. May Grunwald solution: 5 minutes
2. Running water: 1 minute
3. Giemsa stain : 15 minutes
4. Running water: 1 minute
5. Air drying

Dehydration and Clearing of the Smear

After performing Papanicolaou's or hematoxyline and eosin stain the smear is dehydrated by a series of increasing concentration of alcohol. Sudden dipping of the slide in absolute alcohol is detrimental for cell morphology. Removal of alcohol or clearing of the sample is done by putting the sample in xylene. The chemical compound of xylene is dimethyl benzene. The clearing agent should be colorless and its refractive index should be close to the mounting media and coverslip (**Box 16.7**).

Mounting

Mounting medium covers the smear and acts as a permanent bond between the coverslip and the slides. Mounting medium should have the same refractive index of the coverslip and glass slide (**Box 16.8**). The refractive index of the mounting medium should be 1.52 to 1.54. Mounting medium should have a neutral pH to prevent fading of the stain. The viscosity of the mounting medium should be low, otherwise there may be air bubble formation at the time of putting the coverslip. This air bubbles are brownish is color and is known as a cornflake artifact (**Fig. 16.11**).

Coverslip

Coverslip should be wide enough to cover the smear properly. Number 1 coverslip (0.13 to 0.16mm thick) made up of clear

BOX 16.7 Clearing agent

- Xylene commonly used (dimethyl benzene)

Basic properties

- Colorless
- Refractive index close to the mounting media and coverslip.
- It should render the transparent cytoplasm

Caution: Toxic

BOX 16.8 Mounting medium

- It does bonding between the smear and coverslip
- It should have same refractive index of coverslip and clearing agent
- Miscible with clearing agent to prevent optical distortion
- Neutral pH
- Low viscosity to prevent air bubbles formation (corn flakes artifact) during putting coverslip

TABLE 16.3: Comparison of Papanicolaou's stain and MGG stain

	Papanicolaou's stain	*MGG stain*
Nuclear detail	Excellent and very good stain for chromatin stain	The chromatin pattern cannot be studied
Keratin demonstration	Orange G stains keratin as bright orange color	Cannot be demonstrated
Metachromasia	Not a metachromatic stain	Metachromatic stain
Transparency	Transparent stain	Not a transparent stain
Background mucin or necrosis	Not good	Good for demonstration of extracellular substance

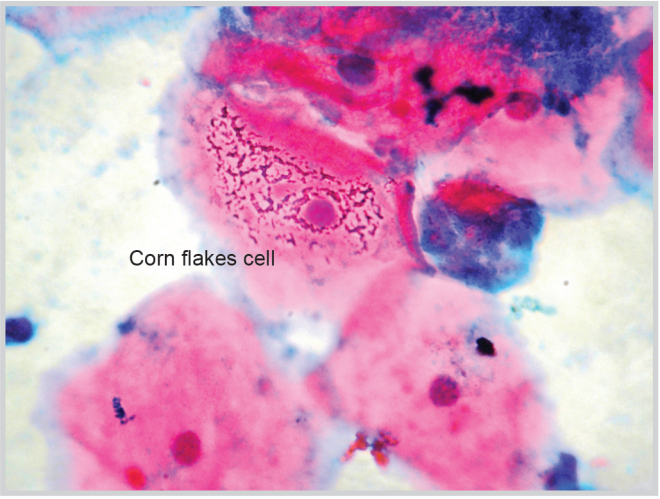

Fig. 16.11: Air bubbles trapped between the coverslip and the glass slide giving rise to cornflakes like cell (Papanicolaou's stain X HP)

glass of 0.130–0.170 mm thickness is preferable. The surface of the coverslip should be plane and the margins should be straight. Coverslipping should be done once at a time to prevent air drying of the smear. It should always be done in properly ventilated well lighted area. Plastic coverslip, liquid coverslip and automated coverslipper are freely available in the market. It is preferable that the mounting medium is dried and the coverslip should be fixed to the slide before examination of the slide (**Box 16.9**).

Storage

After reporting, all the positive slide should be stored properly and should not be discarded. However, the negative slide should be stored for a minimum of 5 years according to rules and legislation of the country.

BOX 16.9 **Coverslipping**

Coverslip
- No 1 glass coverslip of 0.130 to 0.170 mm thickness
- Refractive index: 1.5
- Plane and no irregular surface

Technique
- Put the mounting medium on the coverslip
- Reverse the coverslip and place it on the glass slide over the smear
- Coverslipping area should well ventilated and properly lighted
- Mounting medium should be dried before submission of the slide

Comment: Liquid coverslip and automatic coverslipper are also effective

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CHAPTER 17

Special Stains and Immunocytochemistry

Chapter Contents

INTRODUCTION

Cytochemistry and immunocytochemistry (ICC) are now an integral part of the routine laboratory service. In this chapter, the basic principles, indications and the significance of these stains have been discussed.

SPECIAL STAINS

Special stains are required in addition to routine cytology for various purposes. These are highlighted in **Box 17.1**. The preliminary aim of the special stains is the diagnosis and subtyping of the malignant cells by identifying certain cell products and also diagnosis of various infective agents particularly acid-fast bacteria and fungi.

BOX 17.1 Applications of special stains in cytology

- To demonstrate cellular products for confirmation of the type of malignant cells
  - PAS-Alcian blue with or without diastase in mesothelioma
  - Mucicarmine stains for mucinous adenocarcinoma
  - Melanin for melanoma
  - Oil red O for lipid in renal cell carcinoma
- To demonstrate some extracellular substances with special importance such as amyloid by congo red stain
- To demonstrate infective organisms such as Gram-positive bacteria, acid-fast bacteria or fungi
- DNA stain for DNA content and ploidy.

Periodic Acid Schiff's Stain

Periodic acid Schiff's (PAS) stain demonstrates glycogen. Glycoprotein, sialomucin and neutral mucin are all positive for PAS stain. Periodic acid Schiff's positive material takes deep magenta color stain.

Basic Principle

In this staining reaction, the hydroxyl group attached to the carbon atom in carbohydrate is oxidized to two free aldehyde groups by periodic acid. This aldehyde group reacts with Schiff's reagent and produces deep magenta color. Periodic acid Schiff's positive reaction occurs in glycogen and also monosaccharides, polysaccharides, glycoproteins, mucoproteins, phosphorylated sugars, inositol derivatives and cerebrosides.

Application

Periodic acid Schiff's stain is used to demonstrate glycogen in the cells of adenocarcinoma, demonstration of fungus (capsule) and any mucopolysaccharide substances, basement membrane and mucin.

Steps

- Alcohol-fixed smear to pass through graded lower concentration of alcohol and smear to bring in water
- Periodic acid (1%) for 10 minutes
- Clean with water
- Schiff's reagent for 30 minutes
- Clean in running tap water for 5 minutes
- Counterstain with hematoxylin
- 95% ethyl alcohol
- Absolute alcohol
- Clear in xylene
- Mounting

Mucicarmine Stain

Mucicarmine technique is used to demonstrate mucins particularly acid mucin.

Principle

The active dye molecule in mucicarmine stain is a chelate complex made of cationic aluminum ions and multiring molecule carminic acid. This positively charged chelate complex is known as carmine. Possibly, this positively charged carmine reacts by electrostatic attraction to the anionic groups of acid mucins.

Application

Mucicarmine stain is applied to demonstrate acid mucin in the malignant cells of adenocarcinoma. It also stains capsule of *cryptococcus*.

Steps

- Smears to bring in water
- Hematoxylin for 5–10 minutes
- Wash in water
- Mucicarmine solution 60 minutes
- Wash in water
- Metanil yellow solution for 30 seconds
- Rinse in water
- 95% ethyl alcohol
- Absolute alcohol
- Xylene
- Mount.

Interpretation

Positive mucicarmine stain shows dark red color.

Alcian Blue

Alcian blue stains acid mucin. Acid mucin is a sulfated tissue mucin and reacts with a cationic dye.

Basic Principle

Alcian blue is a group of water-soluble polyvalent basic dye. The dye is made of a copper containing phthalocyanine ring with copper atom in its center. Phthalocyanine ring is also attached with four isothiouronium groups that are positively charged. This positively charged alcian blue dye complex has an attraction with anionic sites of the mucin. Copper imparts the blue color of the dye-mucin complex.

Application

To stain acid mucin present in mesothelioma cells in pleural fluid.

Method

- Rehydration of smear by graded alcohol
- Rinse in water
- Keep the smear in alcian blue for 30 minutes
- Rinse in water
- Counterstain with hematoxylin for 5 minutes
- 95% ethyl alcohol
- Absolute alcohol
- Xylene
- Mount.

Oil Red O

Oil red O stain is used for demonstration of lipid material. The lipid material takes deep red color in this stain.

Application

Oil red O is particularly useful to demonstrate lipid in renal cell carcinoma. The lipoblasts are also oil red O positive.

Steps

- Put the slides directly into oil red O solution for 20–30 minutes
- Rinse with running water
- Counterstain with hematoxylin for 30 seconds
- Rinse in water
- Mounting in glycerin

Preparation of Oil Red O Stain

- Oil red O: 1.2 gm
- Alcohol, 70%: 50 mL
- Acetone: 50 mL.

Feulgen Stain

This stain is specific for DNA and it demonstrates sugar deoxyribose. This is particularly helpful for DNA ploidy examination.

Basic Principle

With the help of hydrochloric acid, the purine-deoxyribose bond is broken and then the exposed aldehyde group is demonstrated by Schiff's reagent. Deoxyribonucleic acid is stained as deep red in color.

Application

Feulgen stain is particularly helpful for DNA ploidy examination.

Steps
- Rehydration of smear by graded alcohol
- Rinse in water
- 1 M hydrochloric acid (preheated at 60°C) for 10 minutes
- Keep in Schiff's reagent for 10 minutes
- Immerse in 0.05 M metabisulfite for 2 minutes three times
- Counterstain by 0.01% fast green
- Dehydrate in absolute alcohol
- Xylene
- Mount.

Congo Red Stain

Congo red stain is specific for demonstration of amyloid material.

Principle

Congo red is an acidic diazo dye consisting of two molecules of naphthenic acid attached to one molecule of benzedrine. The dye molecules bind to amyloid by hydrogen bond and have an oriented arrangement on the amyloid fibrils. This binding of dye-amyloid complex causes absorption of certain wavelengths of light and produces greenish birefringence under polarized light.

Application

Congo red is used to demonstrate amyloid material particularly in abdominal fat pad and medullary carcinoma of thyroid.

Steps
- Slides bring in water
- Hematoxylin stain for 5 minutes
- Alkaline sodium chloride solution for 20 minutes
- Alkaline Congo red for 20 minutes
- Rinse in alcohol
- Dried and mounted

Mycobacterial Staining

Mycobacterial group of organisms are all stained by Ziehl-Neelsen staining.

Basic Principle

Mycobacterial cell wall contains mycolic acid. Mycolic acid greatly influences the penetration and removal of any dye. Phenolic acid and high temperatures increase the porosity of the membrane and helps in the penetration of the dye. Mycolic acid resists the removal of the stain by acid and alcohol.

Application

Demonstration of mycobacteria tubercle and lepra bacilli.

Steps
- Bring the slide in water by treating it with graded alcohol
- Hot carbol fuchsin for 30 minutes
- Two to three dips in acid alcohol for differentiation
- Counterstain with methylene blue for 1 minute
- Clean with water
- Dry

Acid Alcohol

- Concentrated hydrochloric acid: 3 mL
- Alcohol 95%: 97 mL
- *Carbol Fuchsin*
  - Basic fuchsin: 0.5 gm
  - Absolute alcohol: 5 mL
  - 5% aqueous phenol: 100 mL
- *Methylene Blue Stock Solution*
  - Methylene blue: 1.4 gm
  - 95% Ethanol: 100 mL
- *Methylene Blue Working Solution*
  - Methylene blue: 10 mL
  - Water: 90 mL

IMMUNOCYTOCHEMISTRY

Applications

Application of ICC in cytology material has opened a new horizon in this field. The demonstration of various antigenic markers of the cell with the help of monoclonal antibodies reveals the additional character of a cell. Immunocytochemistry on the cytology material provides us all the necessary information that is available from histology sample. Immunocytochemistry technique has been widely used in different areas of cytology (**Box 17.2**).

The cytology samples are easily obtainable and inexpensive to get (**Box 17.3**). However, cytology material is usually not abundant to apply to large panel of antibodies until a cell block is used. Presently, ICC is an indispensable ancillary technology in the field of cytology.

Sampling Techniques for Immunocytochemistry

Sample Collection

Different types of specimen can be used for ICC such as direct smears, cytospin preparations, cell blocks and liquid-based cytology (LBC) preparations (**Box 17.4**).

BOX 17.2 Major applications of immunocytochemistry in cytology

- Diagnosis of malignancy particularly in effusion sample
- Subtyping of lymphomas
- Typing of tumor such as carcinoma, sarcoma, melanoma, etc.
- Hormone or receptor expression such as estrogen, progesterone receptors, HER-2 receptor in breast carcinoma
- Diagnosis of infective organisms

BOX 17.3 Pros and cons of immunocytochemistry on cytology

- Sample: easily obtained, safe, inexpensive
- All the immunological markers can be applied on cytology
- Antigen retrieval not necessary
- Result depends on sample, such as cell block, provides best result
- Scanty cellularity may not allow to use large panel of antibodies.

BOX 17.4 Different cytological preparations

Direct smear
Advantages
- No extra sampling
- No wet preparation

Disadvantages
- Background staining artifact
- Limited antibody panel

Cytospin
Advantages
- Multiple smears can be made
- Panel of antibodies possible
- Overall good result

Disadvantages
- Specimen not fit for bloody and mucoid materials
- Difficult on scanty cellularity
- Best choice for lymphoid neoplasm

Cell block
Advantages
Multiple sections can be made
- Clean background

Disadvantages
- Time consuming

Direct smear: The air dried smear is fixed in formalin and then followed by alcohol. The direct smear is usually made from fine-needle aspiration cytology (FNAC), brush smear or touch smear. The air-dried direct smear is used for ICC when it is not feasible to get extra slides or other preparation. The quality of the immunostain is poor in this type of preparation as there may be severe background artifact after immunostain. A panel of antibodies cannot be used in direct smear. However, individual cell morphology is better seen in direct smear.

Cytospin preparations: Specimens collected in nonfixative fluid, such as FNAC material or fluid specimen such as effusion sample or cerebrospinal fluid (CSF), are used for cytospin centrifugation. Gross blood mixed or mucus mixed materials cannot be used for cytospin. Multiple wet-fixed slides can be prepared from cytospin for application of a panel of antibodies for ICC. Background artifact may be seen but overall cytospin preparation gives good result for ICC.

Cell block: We routinely do ICC on cell block material. It is considered as the best preparation for ICC. Any specimen can be used for cell block except the specimen with very low cellularity such as ureteric urine sample or CSF. There should be a proper standardized practice of making a cell block for ICC. The major advantages of cell blocks are:
- To make multiple sections for a panel of ICC
- To have proper control sections for ICC
- To keep the material for archival use, and
- To have a minimum background staining.

Liquid-based cytology: Liquid-based cytology has now been widely used in different laboratories. Multiple smears can be made by LBC. Other advantages of LBC are clean background and concentrated cells in a small area. Liquid-based cytology preparations provide good results for ICC.[1]

The choice of the above techniques depends on individual laboratory's discretion. Many laboratories prefer cytospin preparation particularly in lymphoid neoplasm. However, in our laboratory, we prefer cell block as it gives many distinct advantages.

Fixation

It is very important to use appropriate fixative for ICC. In fact, the choice of proper fixation plays a crucial role in ICC. Buffered neutral formalin (4%) is probably best fixative for ICC in cytospin, air-dried smear and cell block. Commercially available fixatives are used in LBC preparations and they give equally good results. Various other fixatives are ethanol, methanol and acetone. Overall 95% ethanol is a good fixative for FNAC, brush cytology or cytospin preparations and most of the antibodies work in this fixative.[2] Alcohol fixative is not suitable for the demonstration of HER-2/neu oncoprotein, estrogen and progesterone receptors, and buffered formalin solution is the most desirable fixative in this context.

Control

Proper positive and negative controls are mandatory in the interpretation of ICC staining. Ideally, each laboratory should have proper control of positive slides for each antibody. In case of ICC on cytospin preparation, the laboratory should have a good number of positive cytospin slides in the store. The laboratories who work on cell block only, should have adequate sections from cell block of each positive case. Paraffin embedded sections should not be kept as a control for smear preparation.

Interpretation

Adequate precautions should be taken during the interpretation of ICC. It should be always remembered that no immunostain can confirm the presence or absence of malignancy. Cytomorphology and clinical correlation is mandatory for the diagnosis of malignant lesions. It is always advisable to use a panel of antibody for diagnosis. Certain things such as clinical history, cytomorphology and other biochemical and radiological features should be kept in mind before ordering a panel (**Box 17.5**).

BOX 17.5 Factors to be considered before ordering panel of immunocytochemistry

- Clinical history
  - Age and sex
  - Chief complaints
  - Past history of any disease
  - Radiological features
- Cytomorphological features
- Provisional diagnosis
- Determination of panel

BOX 17.6 Causes of false positive immunocytochemistry

- Poorly fixed cells
- Too high antibody titer
- Acute inflammation in the background
- Crushed cells
- Insufficient blocking of endogenous peroxide
- Less specific antibody
- Necrotic or cell debris in the background
- Various technical problems such as:
  - Omission of steps
  - Use of wrong antibody
  - Drying of smear

BOX 17.7 Causes of false negative immunocytochemistry

- Too low antibody titer
- Fragile cell cytoplasm
- Poorly-fixed cells
- Alcohol-fixed smear for nuclear antigen
- Insufficient retrieval of antigen

There are certain causes of false positive interpretation of ICC such as poorly fixed cells, too high antibody titer, acute inflammation in the background, insufficient blocking of endogenous peroxide, less specific antibody and necrotic or cell debris in the background (**Box 17.6**).

Similarly, one should be careful about false negative ICC. The predominant causes of false negative ICC are highlighted in the **Box 17.7**.

DIAGNOSTIC IMMUNOCYTOCHEMISTRY

Mesothelial Cells Versus Adenocarcinoma

The most common problem in any cytology laboratory is to differentiate mesothelial cells from metastatic adenocarcinoma. Reactive mesothelial cells often show significant pleomorphism and the classical two cell populations may not be evident in distinguishing mesothelial cells from adenocarcinoma. A panel of antibodies is always recommended to resolve the issue.[3,4]

Some important mesothelial markers are discussed first.

Mesothelial Markers

Calretinin: This is a 29 KD calcium-binding protein and is present in the mesothelial cells and also in a wide variety of other cells including steroid producing cells of ovary and testis, renal tubular cells and lipocytes. It is considered as a mesothelial-specific marker and stains both reactive and malignant mesothelial cells. The sensitivity and specificity of calretinin is about 100% and 80%, respectively.[5] Occasionally, the cells of adenocarcinoma may also show calretinin positivity.[6] However, calretinin positivity pattern is less intense and focal in adenocarcinoma cells.

HBME-1: This monoclonal antibody is raised against the cell surface microvillus of the mesothelial cells. HBME-1 stains intensely around the periphery of the mesothelial cells. The specificity of HBME-1 is about 80% and the majority of the mesothelial cells show strong positivity. However, it is also true that a significant population of adenocarcinoma cells also show HBME-1 positivity. Therefore, the utility of HBME-1 is a bit debatable.[7]

Wilms' tumor gene 1: Wilms' tumor gene 1 (WT-1) is present on chromosome 11 and acts as a tumor suppressor. This is a transcription factor isolated in the cells of the kidney. It is expressed in the cytoplasm and nucleus of the mesothelial cells. The WT-1 gene product is also present in desmoplastic small round cell tumors and ovarian serous carcinoma.[8] Therefore, the usefulness of WT-1 is to some extent restricted to differentiate adenocarcinoma from mesothelioma.

D2-40: D2-40 was initially used as a marker of lymphatic endothelial cells. However, it is a relatively sensitive (85%) and specific (95%) marker of mesothelial cells.[9,10]

Cytokeratin 5 and 6: Cytokeratin 5/6 (CK5/6) shows diffuse cytoplasmic positivity in both benign and malignant mesothelial cells. CK5/6 also stains other tumors such as squamous cell carcinoma, breast carcinomas, thymoma and salivary gland tumors.[11]

Adenocarcinoma Markers in Effusion Fluid

Ber-EP4: This antibody reacts with the cell surface glycoprotein of the epithelial cells. This antibody is considered as highly specific and sensitive for adenocarcinoma.[12,13]

Carcinoembryonic antigen: Carcinoembryonic antigen (CEA) is a glycoprotein detected in fetal epithelial cells. It is usually present in a scanty amount in normal epithelial cells and large amount in gastrointestinal adenocarcinoma and lung adenocarcinoma. Specificity of carcinoembryonic antigen (CEA) is very high (100%) and it is considered as a useful marker to differentiate mesothelial cells from adenocarcinoma.[14]

MOC 31: This is a transmembrane glycoprotein present on the epithelial membrane. MOC 31 is positive in all the cases of adenocarcinoma of the lung and many nonpulmonary

adenocarcinomas. Reactive mesothelial cells rarely show MOC 31 positivity. MOC 31 is considered as an effective antibody to distinguish mesothelial cells from adenocarcinoma.[14]

Leu M1 (CD15): Monoclonal antibody to Leu M1 (CD15) specifically demonstrates carcinoma cells. Sensitivity of Leu M1 is low (29%) and it may also show false positivity as a result of high hyaluronic acid content.[15]

Benign Versus Malignant Mesothelial Cells

This is already discussed in effusion cytology chapter. However, it is mentioned here briefly.

Desmin: Strong positivity of desmin indicates reactive mesothelial cells rather than any malignancy. Malignant mesothelioma cells do not show desmin positivity.[16]

Epithelial membrane antigen: Mesothelioma shows strong epithelial membrane antigen (EMA) positivity. Reactive mesothelial cells show only focal weak positivity of EMA.[17]

Glucose transport protein-1: Glucose transport protein-1 (GLUT-1) is a member of facilitative family of glucose transporter in the cell membrane. This is overexpressed in a variety of neoplasms such as mesothelioma, breast carcinoma and ovarian surface epithelial tumors. The GLUT-1 expression is positive mainly in mesothelioma. The normal benign mesothelial cells are negative for GLUT-1.[18] Therefore, it is claimed as a good marker to differentiate mesothelioma versus normal mesothelial cells.[19]

Epithelial Markers

Cytokeratin: There are three types of cytoskeletal filaments in the cell, namely microfilaments, intermediate filaments (IFs) and microtubules. The IFs are intermediate in size and organize the internal three-dimensional structure of the cell. There are seven varieties of IF and CK is one of them. Cytokeratin is expressed in all types of epithelial cells. Depending on their molecular weights, CKs are classified into 20 different types. The demonstration of a specific CK is immensely helpful in pointing out the origin of an unknown metastatic tumor. Cytokeratin 7 and CK 20 phenotyping are particularly helpful to find out the primary tumor in metastasis.[20] **Table 17.1** illustrates the expression of CK and probable origin of tumors. Stable and consistent expression of CK is very important for diagnosis. Loss or gain of antigen during metastasis or development of the tumor may hamper their potential use. Cytokeratin 20 is stably expressed in colorectal carcinoma, ductal and lobular carcinoma of breast, transitional carcinoma, endometrial, prostate and renal cell carcinoma. Cytokeratin 7 positivity is consistently noted in transitional carcinoma of bladder, biliary adenocarcinomas, lung, ovarian, endometrial, breast and pancreatic adenocarcinomas. Therefore, combined use of CK7 and CK20 immunostain may indicate the probable origin of metastatic tumor of unknown primary cases (**Table 17.1**).

Epithelial membrane antigen: This epithelial marker has been discussed previously.

Carcinoembryonic antigen: This epithelial marker has been discussed previously.

TABLE 17.1: Expression of cytokeratin and probable origin of tumors

Cytokeratin 7	*Cytokeratin 20*	*Tumors*
Positive	Positive	Pancreatic carcinoma, transitional carcinoma of bladder
Positive	Negative	Lung, breast, ovary, mesothelioma
Negative	Positive	Colonic, gastric, merkel cell carcinoma
Negative	Negative	Prostatic carcinoma, hepatocellular carcinoma, renal cell carcinoma, adrenocortical carcinoma

Mesenchymal Markers

Vimentin: Vimentin is an IF which is usually expressed in mesenchymal cells such as fibroblast, endothelial cells, smooth muscle, etc. However, it has been demonstrated in epithelial neoplasms, such as renal cell carcinoma and also mesothelial cells. Therefore, vimentin has limited specificity in the diagnosis of mesenchymal tumors.

Desmin: This is also a variety of IF and present in smooth muscle, skeletal muscle and cardiac muscle.

Actin: Actin is a contractile protein and is present in smooth muscle cells and myofibroblasts.

Neuroendocrine Markers

Chromogranin: Chromogranin is present in the neurosecretory granules in the neuroendocrine cells. It is a reliable marker in all types of neuroendocrine tumors.

Synaptophysin: This is a transmembrane glycoprotein and is present in the presynaptic vesicles of neurons. Synaptophysin is a marker of neuroendocrine and neuroectodermal tumors.

S-100 protein: This is a calcium-binding protein and is present in the nucleus and cytoplasm of glial and Schwann cells. Other than the cells of neural origin, S-100 protein is also demonstrated in melanocytes, Langerhans cells and myoepithelial cells, and the neoplasm derived from these cells.

Lymphoid Markers

The detailed immunophenotyping of lymphoid markers is very useful for identification of lymphoid cells and subclassification of lymphomas. The ICC should be done either on cell block sections and if not possible then on cytospin smear (**Figs 17.1** to **17.3**). Presently flow cytometric immunophenotyping has largely replaced ICC.

CD45 or leukocyte common antigen is the common marker of lymphoid cells (**Box 17.8**). The majority of the cases of non-Hodgkin's lymphoma (NHL) are positive for CD45 except anaplastic large cell lymphoma and plasmacytic lymphoma.

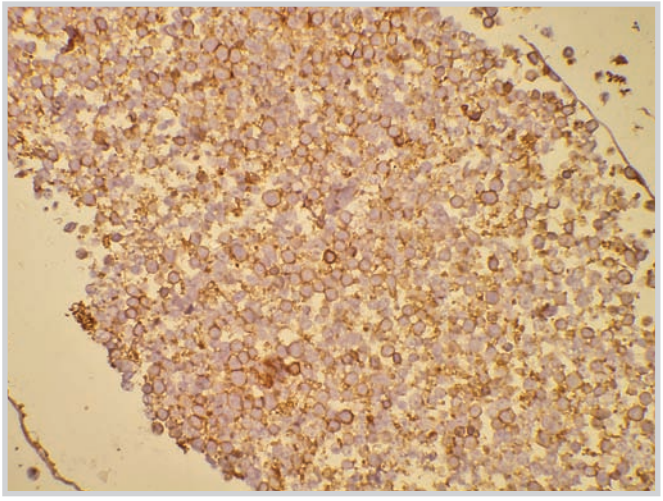


Fig. 17.1: CD45 (leukocyte common antigen) positivity in a case of non-Hodgkin's lymphoma in cell block section (Immunocytochemistry X MP)

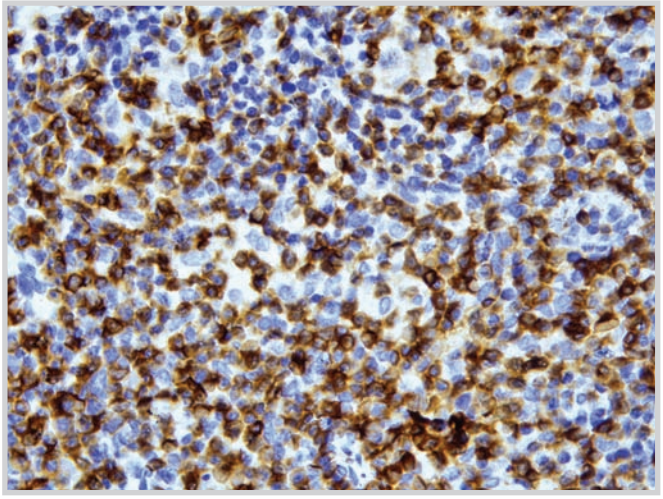


Fig. 17.3: CD3 (T cell marker) positivity in a case of T cell type of non-Hodgkin's lymphoma in cell block preparation (Immunocytochemistry X MP)

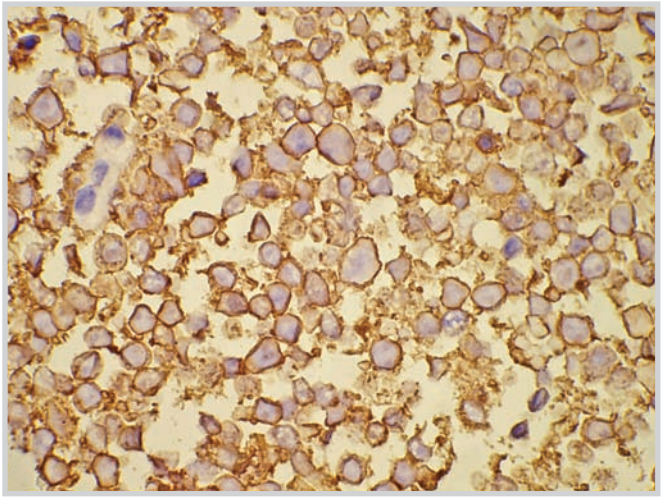


Fig. 17.2: CD20 (B cell marker) positivity in a case of B cell type of non-Hodgkin's lymphoma in cell block preparation (Immunocytochemistry X HP)

The immunophenotyping of different subtypes of B cell NHL has been highlighted in **Table 17.2**.

Melanoma Markers

HMB45: HMB45 identifies an oncofetal glycoconjugate associated with immature melanosomes. This is a highly specific marker of melanoma. However, it also identifies neural crest-derived tumors, occasional carcinomas and immunoblastic lymphomas.[21,22]

Melan A (Mart-1): This is a melanocyte-associated marker with high sensitivity for detection of melanoma. However, Melan A is also positive in peripheral nerve sheath tumor, angiomyolipoma and steroid producing cells of adrenal cortex.[23]

BOX 17.8 Panel of antibodies for lymphoma subtyping

Common lymphoid marker: CD45 (Leukocyte common antigen)
Monoclonality
- Light chain restriction: predominant Kappa or Lambda chain restriction for B cell clonality.

B cell markers: CD19, 20, 23, 10
T cell markers: CD3, 5, 4, 8
Hodgkin's lymphoma: CD15, CD30
NK cell: CD3+, CD56+ and CD19; CD10, CD23 negative

TABLE 17.2: B cell non-Hodgkin's lymphoma subclassification

	CD10	*CD5*	*CD23*	*CD22*	*CD19*	*CD20*
SLL	-	+	+	+	+	+
MCL	-	+	-	+	+	+
FCC	+	-	Variable positive	+	+	+
DLBCL	-	-	-	+	+	+
MALT				+	+	+
Burkitt's	+	-	-	+	+	+

Abbreviations: SLL, Small lymphocytic lymphoma; MCL, Mantle cell lymphoma; FCC, Follicular center cell lymphoma; DLBCL, Diffuse large B cell lymphoma; MALT, Mucosa-associated lymphoid tumor

Germ Cell Markers

Placental alkaline phosphatase: Placental alkaline phosphatase (PLAP) is expressed in normal placenta. This is an oncofetal antigen and is expressed in all germ cell tumors.[24] Placental alkaline phosphatase positivity is seen on the cell membrane and occasionally in the cytoplasm. Therefore, PLAP stain is used to differentiate germ cell tumor and any other undifferentiated malignancy.

Human chorionic gonadotropin: Human chorionic gonadotropin (HCG) is normally secreted by the syncytiotrophoblast cell of the placenta. The beta subunit of HCG is hormone specific. Human chorionic gonadotropin usually shows intense positivity in choriocarcinoma.

DIAGNOSIS OF UNDIFFERENTIATED MALIGNANCIES

Immunocytochemistry is a great help in diagnosis of an undifferentiated malignancy. A judicious panel of IC should be used for determination of the type of the tumor.

A panel of CK, CD45, Vimentin, HMB45 and chromogranin could be used for initial determination of a broad category of the tumor. Once the tumor is classified in broad category then a further detailed ICC could be done for more accurate subtyping and subclassification.

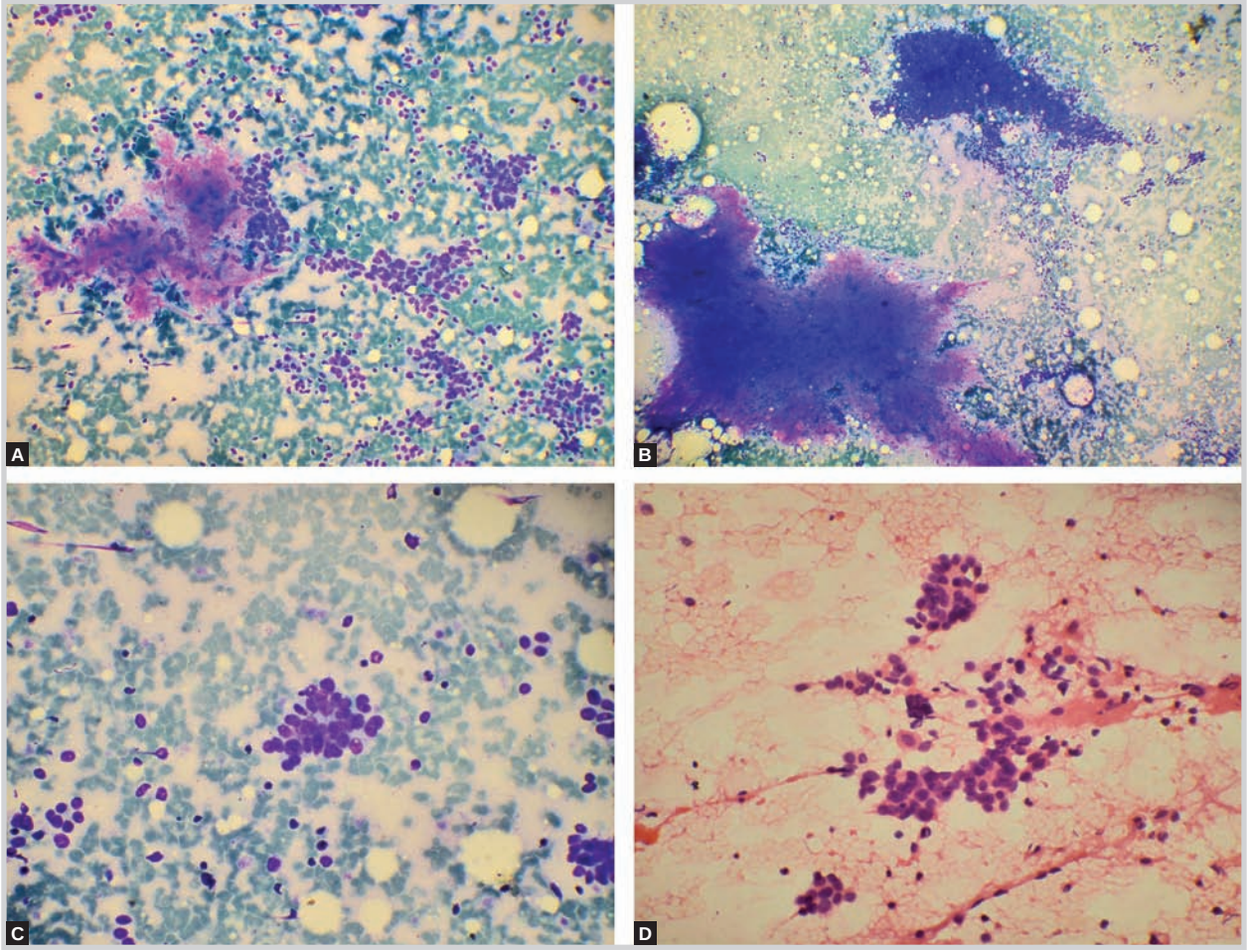
Round Cell Malignancies

Malignant small round cell tumor is a great diagnostic problem in routine cytology sample. Immunocytochemistry is of great help in distinguishing different malignant round cell tumor (MRCT) (**Figs 17.4** to **17.6**). **Table 17.3** shows the ICC pattern of different MRCT.

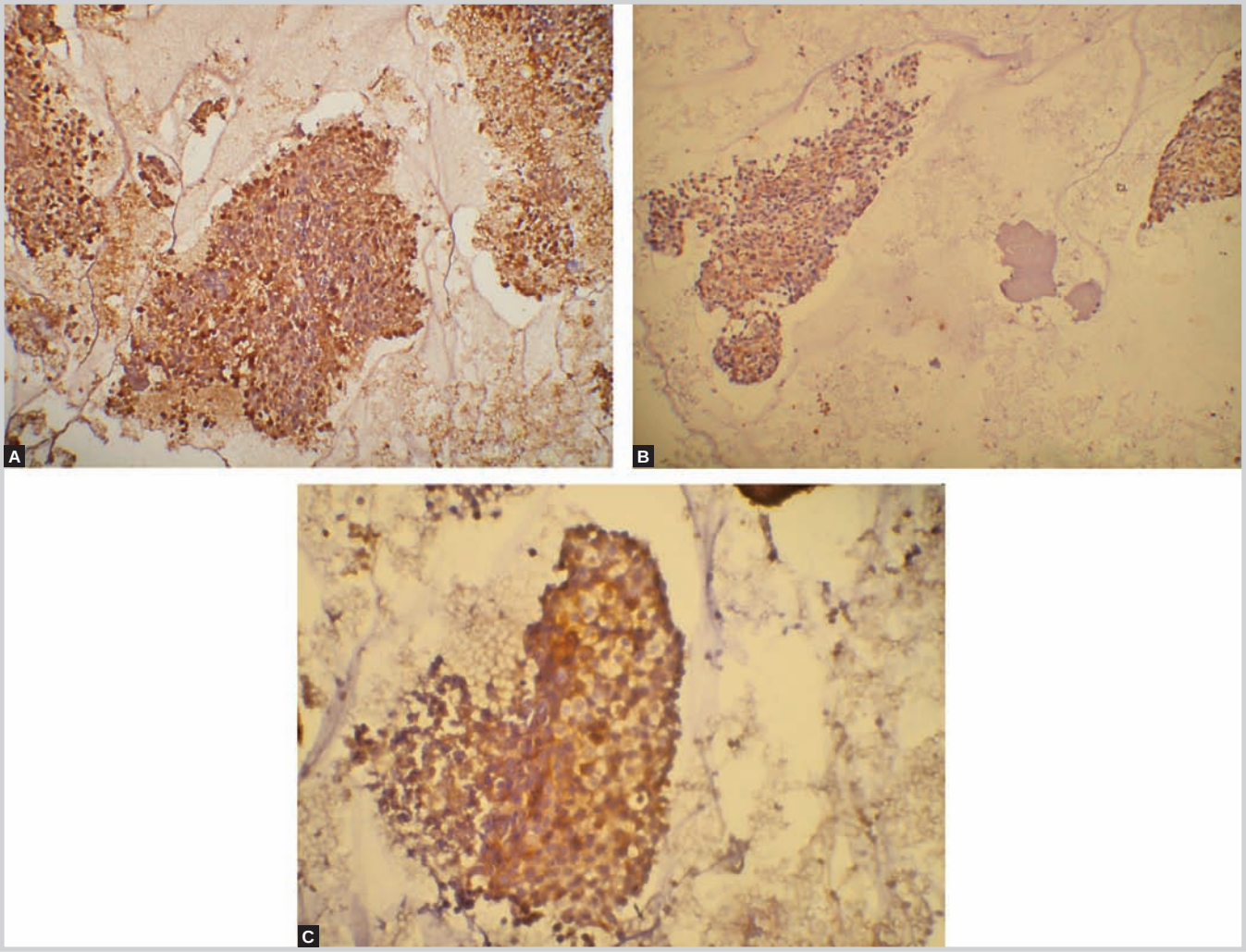
It is important to remember that there is a lack of specificity in ICC and there is also overlapping positive reaction of different monoclonal antibodies. Moreover, some MRCTs may be so poorly differentiated that they may not show any antigenic expression.[25] It is always preferable to apply a panel of monoclonal antibodies for accurate diagnosis.

Problems in Lung Carcinoma

It is very essential to differentiate small cell carcinoma of lung from non small cell carcinoma. Small cell carcinoma of the lung



Figs 17.4A to D: Desmoplastic small round cell tumor. (A) Fine needle aspiration cytology smear shows multiple small clusters and discrete round cells along with stromal fragment (May-Grünwald-Giemsa stain X MP); (B) Fine needle aspiration cytology smear shows large chunk of stromal fragment (May-Grünwald-Giemsa stain X MP); (C) Fine needle aspiration cytology smear shows round cells with scanty cytoplasm and predominantly monomorphic nuclei (May-Grünwald-Giemsa stain X HP); (D) Fine needle aspiration cytology smear shows round cells with hyperchromatic nuclei. Many vague rosette-like structures are noted (Hematoxylin and Eosin stain X HP)



Figs 17.5A to C: Desmoplastic small round cell tumor. Immunocytochemistry from the cell block sections show (A) cytokeratin; (B) vimentin and; (C) desmin positivity. Cytomorphology along with these immunostains is diagnostic of desmoplastic small round cell tumors (Immunostains X MP)

is positive for neuron-specific enolase (NSE), chromogranin and cytokeratin. Presently, due to change of therapeutic approach of non small cell lung carcinoma, it is necessary to differentiate squamous cell carcinoma from adenocarcinoma on cytology specimen. It has been shown that a minimum panel of p63, thyroid transcription factor (TTF) and CK5/6 may be helpful in this aspect (**Table 17.4**). Squamous cell carcinoma is almost always p63 positive and TTF negative, whereas adenocarcinomas are TTF positive and p63 negative. In addition, squamous cell carcinomas are also positive for CK5/6.[26]

Site-specific Antibody in Different Epithelial Malignancies

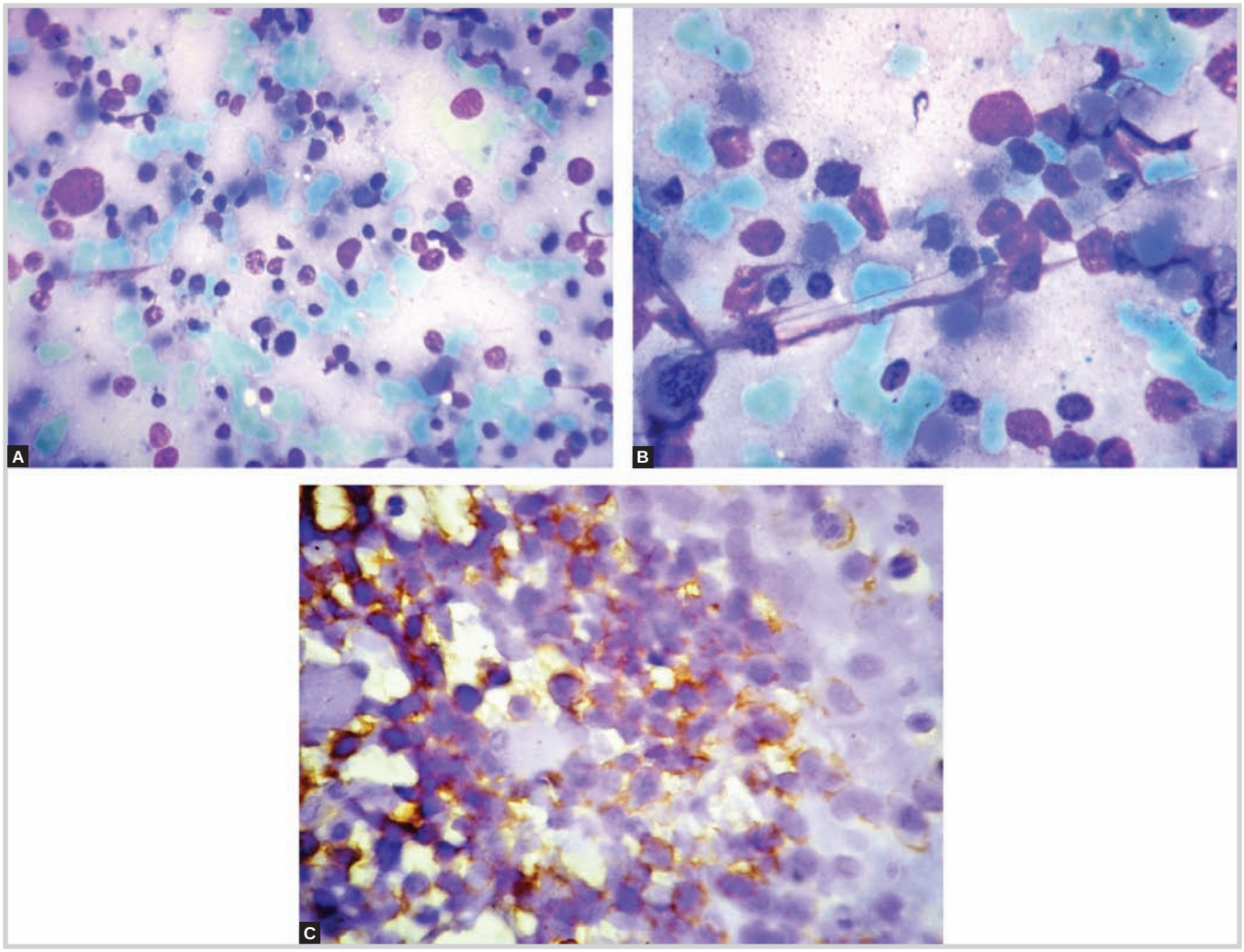
It is often essential to diagnose primary malignancy from a metastatic site or ascertain the exact character of two simultaneously evolved malignancies. Immunocytochemistry is a great help to resolve these problems (**Table 17.5**).

Cytokeratin 7 and cytokeratin 20: The combined use of these two antibodies is very helpful for assessment of the probable site of origin of the epithelial malignancies (**Table 17.1**).

Prostate-specific antigen and androgen receptor: Prostate-specific antigen (PSA) and androgen receptors are helpful in diagnosis of prostate carcinoma. Prostate-specific antigen is positive in normal, hyperplastic and neoplastic prostate tissue. However, PSA is also positive in breast carcinoma and salivary gland tumors.[27,28]

Androgen receptor: Androgen receptor (AR) is also demonstrated in the nucleus of the prostate epithelial cells and it is considered as a reliable indicator of prostate origin of cells. However, salivary gland tumor also shows AR positivity.[29]

Thyroid transcription factor: Thyroid transcription factor (TTF) is a tissue-specific transcription factor and is expressed in follicular epithelial cells of the thyroid, bronchial epithelial cells of lung and adenocarcinomas developed from those cells. Thyroid transcription factor is occasionally expressed in ovarian neuroendocrine carcinomas and carcinoid of lung.[30]



Figs 17.6A to C: (A) 10 cm D swelling in the right gluteal region in a 11-year-old boy. Smear shows discrete round to oval cells (May-Grünwald-Giemsa stain X MP); (B) High powered view of the same. Cells are round with scanty cytoplasm and fine chromatin (May-Grünwald-Giemsa stain X HP); (C) Cell block section shows strong MIC-2 positivity. Tumor cells are negative for CD45, desmin and cytokeratin. Based on immunocytochemistry and cytomorphology, the tumor was diagnosed as peripheral neuroectodermal tumor (MIC-2 stain X HP)

TABLE 17.3: Malignant round cell tumor

	Immunocytochemistry						
	CD45	***NB-84***	***Myo D1***	***CK***	***Desmin***	***WT-1***	***MIC-2 (CD99)***
NHL	+	-	-		-	-	-
NB	-	+	-	-	-	-	+
EWS/PNET	-	occasional	-		-	+	+
WT	-	+	-	+	+	Focal	-
RMS	-		+		+	+	-
DSRCT	-		-	+	+	+	
Synovial sarcoma	-	No data	No data	+	-	-	Occasional

Abbreviations: NB, Neuroblastoma; EWS, Ewing's sarcoma; PNET, Peripheral neuroectodermal tumor; WT, Wilms' tumor; RMS, Rhabdomyosarcoma; DSRCT, Desmoplastic small round cell tumor; NHL, Non-Hodgkin's lymphoma; CD, Cluster of differentiation; CK, Cytokeratin

TABLE 17.4: Immunocytochemistry panel of lung carcinoma

Type of carcinoma lung	*Immunocytochemistry*				
	P63	*TTF*	*NSE*	*Chromogranin*	*CK5/6*
Small cell carcinoma	-	-	+	+	+
Squamous cell carcinoma	+	-	-	-	+
Adenocarcinoma	-	+	-	-	-

Abbreviations: TTF, Thyroid transcription factor; NSE, Neuron-specific enolase
+ means positive
- means negative

TABLE 17.5: Site-specific antibody

Organ or cell type	*Antibodies*
Thyroid	TTF, Thyroglobulin
Breast	Estrogen and progesterone receptors, EMA
Lung	TTF, CK7 positive and CK20 negative
Pancreas	CK7 and CK20 positive, CA19-9
Liver	Heppar, Alpha fetoprotein, CK7 and CK20 negative
Colon	CEA, CK7 negative, CK20 positive
Mesothelial cells	Calretinin, WT-1, CK5 and 6 positive
Prostate	PSA, AR, CK7 and CK20 negative
Bladder	CK7 and 20 positive

Abbreviations: CEA, Carcinoembryonic antigen; WT1, Wilms' tumor; EMA, Epithelial membrane antigen; PSA, Prostate-specific antigen; AR, Androgen receptor ; TTF, Thyroid transcription factor; CK, Cytokeratin

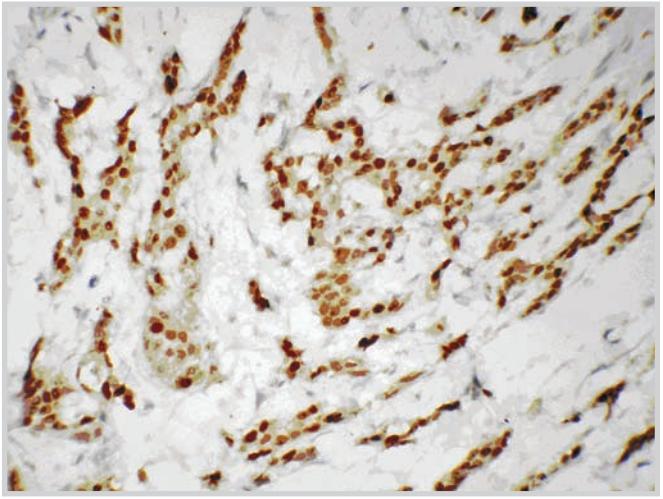


Fig. 17.7: Estrogen receptor positivity in a carcinoma of breast in cell block section. The cell block is made from cell scrape of the cytology smear (immunostain X MP)

Estrogen and progesterone receptors: These two hormonal receptors present in the nucleus of the breast (**Fig. 17.7**) and endometrial adenocarcinomas. They may help in identification of metastatic carcinoma of the breast in selective conditions.

IMMUNOCYTOCHEMISTRY FOR THERAPY AND MANAGEMENT

Molecular targeted therapy is a type of therapy where the drugs or other chemicals block the growth and progression of cancer cells by interfering with the specific molecules related to the growth and progression of cancer cells.[31]

The different antigen receptors can be accurately assessed in the cytology specimen.[32]

Estrogen and progesterone receptors: Estrogen receptors and progesterone receptors are nuclear receptors and can be successfully assessed on cytology specimen. Estrogen receptors/progesterone receptors nuclear positivity is related to antiestrogen hormone therapy of breast cancer.

HER-2/neu: This oncogene is overexpressed in a certain fraction of breast carcinoma. Overexpression of HER-2/neu is manifested as intense staining of cell membranes. The status of HER-2/neu overexpression in breast cancer is needed to know for administering antiHER-2/neu antibody.

CD117 (C-Kit): CD117 is also known as C-kit. It is a cell membrane protein and is expressed in gastrointestinal stromal tumor (GIST), primitive neuroectodermal tumor, seminomas and melanomas. The demonstration of the CD117 expression is necessary for the diagnosis of GIST.[33]

The GIST often shows the mutation of C-Kit or CD117. This results in high surface level of CD117 expression. Gastrointestinal stromal tumor with C-Kit mutation is responsive to tyrosine kinase inhibiting agent (imatinib) therapy.[34]

ANTIBODIES DIRECTED TO ANTI-INFECTIVE AGENT

Various antibodies can be used to identify the infective organisms on cytology smear. Antibodies to *Cytomegalovirus*, *Chlamydia*,
Herpes simplex virus, and human papillomavirus are available. Epstein-Barr virus (EBV)-related antigen is useful to detect the presence of EBV.

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CHAPTER 18

Light Microscope

Chapter Contents

VISIBLE LIGHT

Visible light covers the narrow range of the electromagnetic spectrum extending from deep violet to red that means 400 nanometer wavelength to 750 nanometer wavelength of light. Ordinary white light is the mixture of light of different wavelengths. Monochromatic light is the light of a single wavelength.

IMAGE FORMATION IN HUMAN EYE

Light from the object passes through the iris, cornea and lens. Ultimately, the light is focused on the retina and through the optical nerve, the image is transmitted to the brain for final interpretation (**Fig. 18.1**).

Lens

Human eye is not able to magnify the image of the object. Simple convex lens as glass magnifiers can magnify the image of an object. The convex lens if kept between the object and the eye and is focused properly then the magnified virtual image is formed towards the object side (**Fig. 18.2**). In fact, this was the basic principle of early light microscope.

HISTORY

Long back about 3000 years ago, lens was used by the Assyrians as burning glass. Egyptian and Greeks have also used biconvex lenses about 800–400 years BC. History says that the famous Roman Emperor, Nero, used emerald lenses to watch the gladiator games. In the year 1590, two Dutch opticians Hans and Zacharias

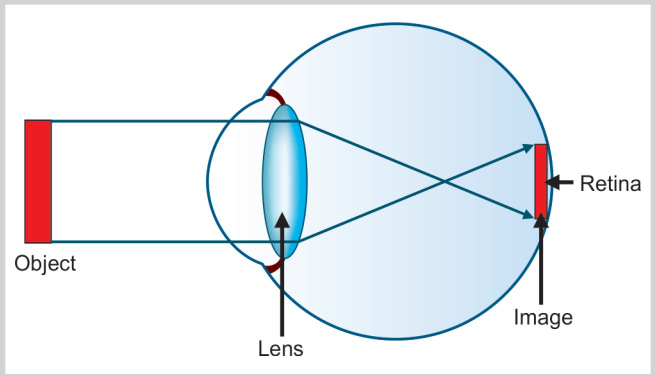

Fig. 18.1: Schematic diagram shows how the image is formed in the eye

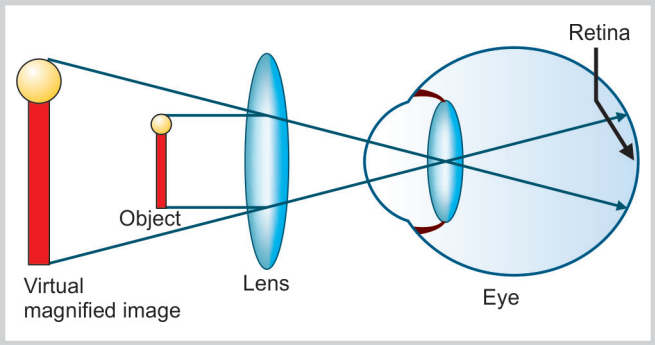

Fig. 18.2: Schematic diagram shows how the image is magnified if a convex lens is kept between the object and eye. A magnified virtual image is formed towards the side of the object

Janssen (father and son) invented compound microscope. This was further improved by Anton von Leeuwenhoek from Holland in the 17th century. He noted blood cells, sperm, ameba and other various microorganisms in detail. Today microscopy system is much more advanced and much more complicated. Modern microscope can not only use light, but can also use electron beam to produce a magnified image with very high resolution.

LIGHT MICROSCOPE

Light microscope uses visible light for the source of light so it is named as light microscope. The light microscope performs three basic tasks: magnification, resolution and contrast.

Magnification

It is one of the basic tasks of a light microscope. In most of the microscope, the first magnification is done with the objective and this image is further magnified by an ocular lens. Therefore, the total magnification is: magnification done by the objectives multiplied by magnification done by ocular lens.

Resolution

Resolution means the finer details of the object and it is defined as the ability to distinguish two closely spaced objects. The resolution of a microscope depends on two factors: (1) wavelength of light and (2) the light accumulating power of the objective and condenser lenses. This light gathering capability of the lens is mathematically represented as numerical aperture (NA). NA value is usually written on the objective. If the resolution is expressed as the smallest distance separating the two closely spaced very small objects (d_{min}),
then
$d_{min} = 0.61\delta/NA$
Sigma is the wavelength of light.

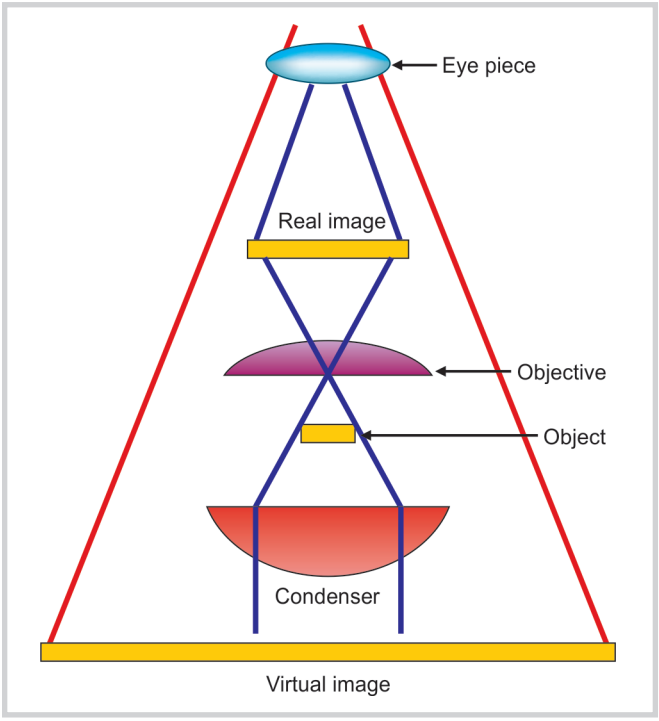

Fig. 18.3: Schematic diagram explains the basic principle of the light microscope. Final magnified virtual image is formed beyond the condenser

IMAGE FORMATION IN A COMPOUND MICROSCOPE

The microscope is the simple collections of lenses to magnify the image of an object. The first lens is the objective which creates a real image in the body tube of the microscope and the second lens is the eye piece that further magnifies the first image. At first, light passes through the condenser that concentrates the light in a collection of parallel beams onto the object placed on the stage. The light subsequently passes through the objective which projects a real inverted image within the body tube of the microscope. The eye piece second time magnifies the real image and produces a virtual image that appears to be 250 mm away from the eye (**Fig. 18.3**).

Mechanical and Electrical Components

Base: This is the bottom support of the microscope.

Stage: This is the flat movable platform like area where the object is kept. The stage can be moved side by side, also up and down.

Tube: It is the part that connects the eyepiece to the objective of the microscope.

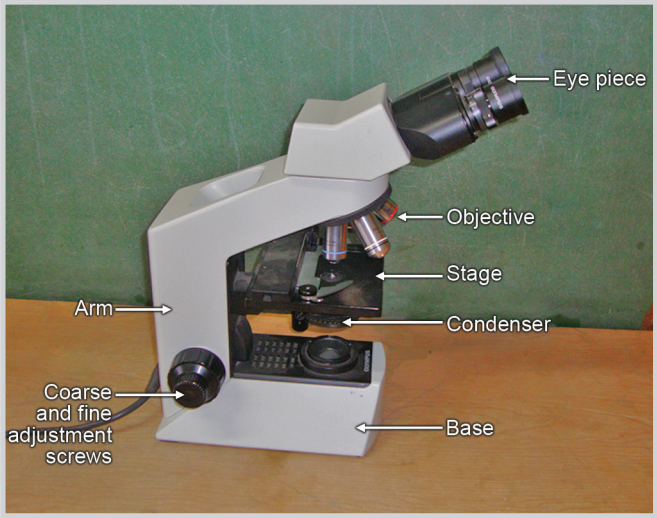

Fig. 18.4: Simple light microscope and its parts

Arm: It is the connecting part between the tube and the base of the microscope.

Revolving nose-piece: This is the part that holds the different objectives of the microscope for different magnification of the image of the object.

Coarse and fine adjustment knobs: These knobs help to move the stage up and down.

Diaphragm: It is situated just below that stage and it regulates the amount of light on the specimen.

Light source: Light is projected below and goes upwards through the diaphragm and the object and lenses up to the eye (**Fig. 18.4**).

Optical Components

The optical components of the light microscopes are:
Condensers: The principal function of the condenser is to focus light on the object placed on the stage. This is particularly helpful when the higher objective lens is used.

Objectives: There are multiple objectives attached to the rotating nose piece. The main function of the objective is to magnify the image and to produce a real inverted image in the body tube of the microscope.

Eye piece: It contains the ocular lens. This ocular lens further magnifies the real image when it is focused on it. A virtual image is produced at 250 mm away from the eye.

Care and Handling of the Microscope

- During transport, the stage of the microscope should be parallel to the ground. One hand should always support the base and the other arm should grasp the arm of the microscope.
- The lens of the microscope should not be touched with the hand. The oil immersion lens should always be cleaned by the lens paper after use.
- Lens are delicate so over cleaning may damage the lens. If it is necessary to clean, then compressed air can be used for cleaning the lens.
- After the work, the nose piece should always be rotated to keep in low power objective and far away from the stage.
- The microscope should always be covered by a dust cover plastic sheet.

FLUORESCENCE MICROSCOPE

The fluorescence microscopy uses light of much higher intensity instead of ordinary light in the light microscope. This high intensity light excites the fluorescent species in the object of interest and emits lower energy light that forms an image detected by the observer.

Principles of Fluorescence

When light of high energy hits the atom then the electron shifts from lower orbit to higher orbit and immediately the electron again jumps back to the ground state and releases photon or packet of light. This makes the atom fluorescing.

Principle of fluorescence microscopy: The basic principles of fluorescence microscopy are described below:

- The specimen or object is stained with a fluorescent dye
- The light of the high energy excitation beam is directed through the filter to the object
- The fluorescent dye bound object is hit by the excitation beam of light and emits light of longer wavelength (low energy light)
- This emitted light travels through the filter to the observer and the image is visualized.

Incident fluorescent light: The **Figure 18.5** explains the basic working principles of fluorescence microscopy. In the incident

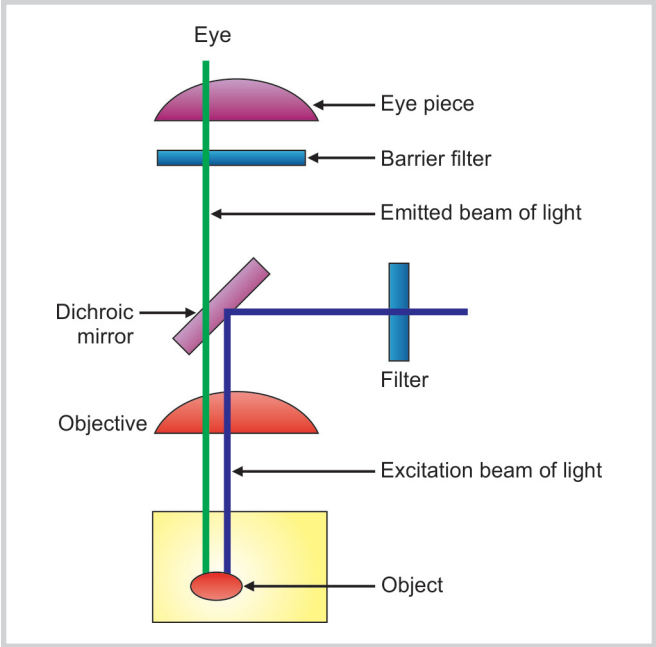

Fig. 18.5: The schematic figure explains the basic working principles of incident fluorescence microscopy

fluorescent light microscopy, the high energy light beam passes directly through the filters and then with the help of a dichroic mirror it is directed towards the objectives followed by the object. This excitation light beam hits the fluorescent dye bound object and low energy light is liberated. The emitted light passes through the objective and then with the help of a dichroic mirror, it is transmitted through the barrier filter to the eye of the observer. The barrier filter blocks any unwanted excitation wavelength. The dichroic mirror has the unique property to transmit light of some wave length and it reflects the light of another wavelength. Here, the dichroic mirror reflects the excitation beam of light and it allows transmitting emitted beam of light.

Transmitted light fluorescent (**Fig. 18.6**): Here, light from the source passes through the heat absorbing filter followed by red light removing filter that stops the red light and then through the other filter that selects the only excitation light beam. The excitation beam passes through the condenser to the object of interest. The objective collects both the excitation and emitted wave length. The barrier filter allows only the emitted beam to pass through to the eye of the observer.

Application

- Fluorescent microscope is used for the detection and imaging of the specific component of the cell or an object
- It is also used for DNA or RNA study
- Fluorescent in situ hybridization technique can show cytogenetic abnormalities of the cell.

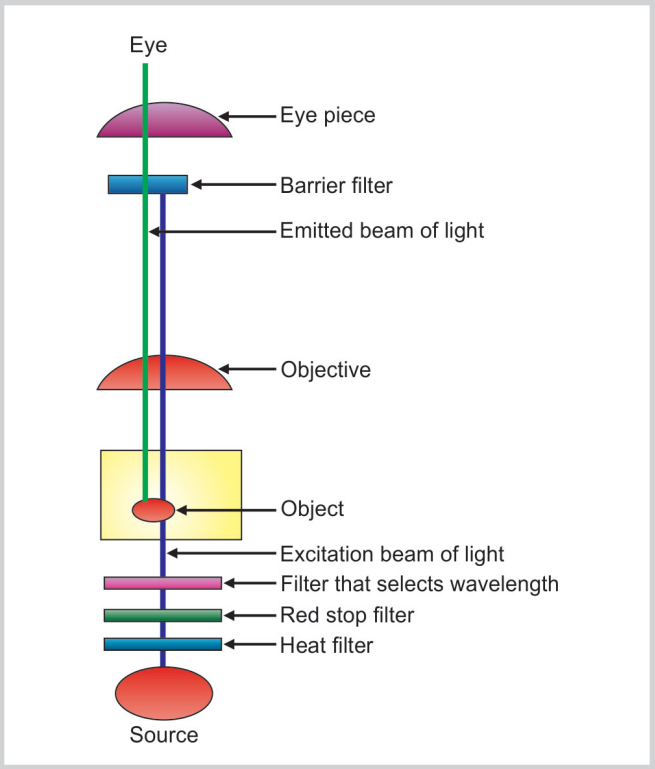

Fig. 18.6: The schematic figure explains the basic working principles of transmitted light fluorescence microscopy

CHAPTER 19

Digital Image Analysis

Chapter Contents

INTRODUCTION

Perception of human being is subjective. However, we need objective and quantitative assessment in our day-to-day cytology practice. Image analysis helps us to have quantitative cytology. The term image analysis means acquiring and then further analyzing the quantitative data from any image of an object. In the last two decades, there has been a massive development in the field of image analysis. This is due to significant computational capability for data accumulation and storage. The other factor is rapid development of knowledge based systems for data analysis and diagnostic decision making. Presently image analysis is used not only as a major research tool in the field of cytology, but also has been applied routinely in various fields such as in automated screening technology of cervical cancer screening.

BOX 19.1 **Image analysis: Few basic points**

Steps:
- Digitalization: Image is converted into digits depending on gray value
- Detection: Object of interest is selected by adjusting gray value or color code
- Editing: Detected image is processed to extract data
- Feature extraction: The data is extracted from the identified object

Performing image analysis: Interactive and automated
Basic requirements: Microscope, digital camera and computer
Types: Morphometry, cytometry and pattern recognition

PRINCIPLES OF IMAGE ANALYSIS

The knowledge of principles of image analysis (**Box 19.1**) is very essential for understanding and further application of this field. The basic principles and steps are described below.

Image Digitalization

In this step, the image is stored in the computer as digital image. The smallest unit of the image is known as a pixel. Each pixel is located in particular X and Y axis. Now depending on the gray intensity of the pixel of the black and white image the pixel is given a particular numerical value. So every pixel of the image in each particular location (X, Y) is stored in the computer as a numeric value. Now, from the stored gray value data, the computer can generate the whole image again. In case of colored image, each pixel is given three values for red, green and blue depending on the relative intensity of the image (**Fig. 19.1**).

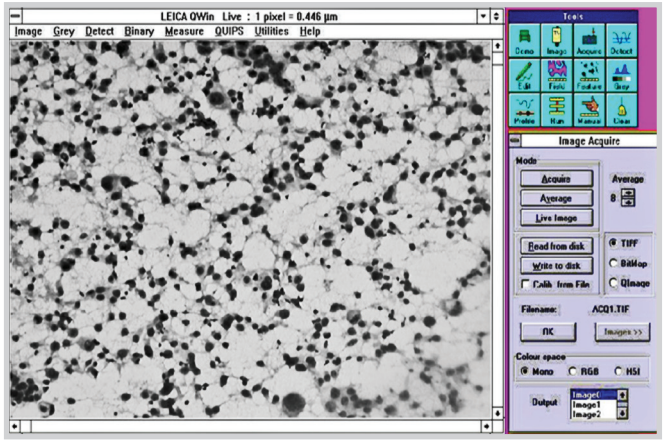

Fig. 19.1: At first the image is digitized. This digitized image may be black and white or in color

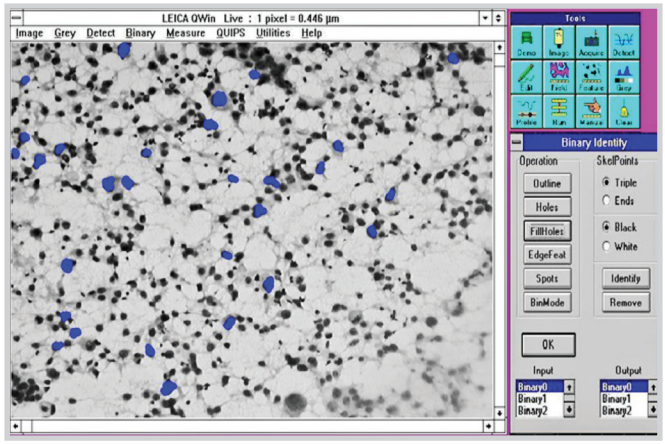

Fig. 19.2: Object of interest is detected by adjusting gray value

Image Detection

Now, from the digital image stored in the computer, one can detect the area or object of interest by manipulating or adjusting the gray value or color code of the image (**Fig. 19.2**).

Image Editing

Image editing is needed to process the detected object of interest so that it is free of background noise. The detected object should also be free of any overlapping objects. Different mathematical algorithms can be used to separate or segregate the objects.

Feature Extraction

Feature extraction means extraction of different geometric data from the object such as size, shape, texture, etc. In each case, hundreds of different features can be measured (**Fig. 19.3**).

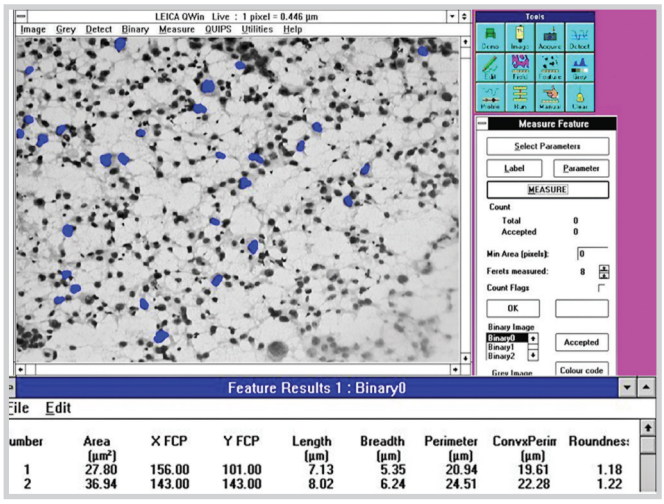

Fig. 19.3: Data is extracted from the detected image

INSTRUMENTS REQUIREMENTS

The basic instruments required are: (1) microscope, (2) attached digital camera, (3) central processing unit, (4) keyboard, (5) mouse pointer and (6) a suitable software program.

A digital camera is attached to the microscope. The image is transferred from the digital camera to the computer processing unit and is shown directly into the monitor. Now, this digitized image is manipulated for data extraction and analysis (**Fig. 19.4**).

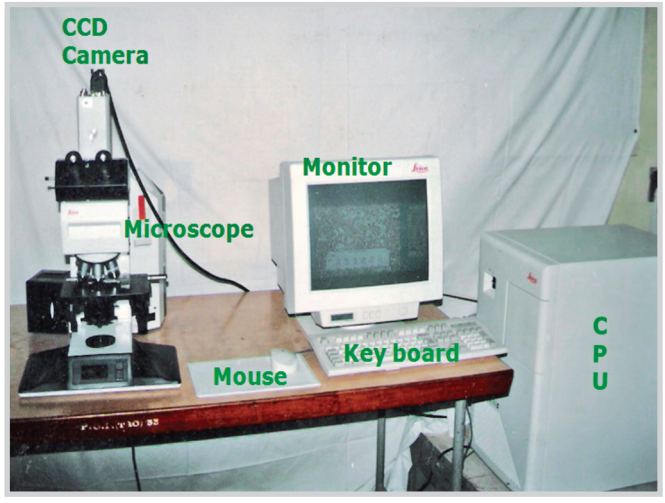

Fig. 19.4: Basic instruments of image cytometry are highlighted in this photograph

IMAGE ANALYSIS: AUTOMATED OR INTERACTIVE

An automated image analysis system has automated control of magnification, illumination, movement of scanning stages and fine focus. All these functions are controlled by means of a microcomputer. The interactive image analysis features a user friendly mode wherein the user can select the cell of interest and

obtain various cell parameters required. The system is equipped with a microscope and a video camera for acquisition of cell images. The image processing hardware accepts the video data from the camera and converts the image into the digital image and then into signals for displaying the image on the monitor. Now, with the help of mouse the object of interest is selected, edited and features are extracted.

TYPES OF IMAGE ANALYSIS

It can be arbitrarily classified as:

- Morphometry
- Cytometry
- Chromatin pattern recognition

Morphometry

This means a quantitative description of geometric features of structures of any dimensions. It is based on analysis of two-dimensional (2D) structure. Three-dimensional (3D) (stereologic) information is derived from these 2D data, either by making assumptions about the 3D structure regarding object size and shape or by directly calculating the third dimension.

The targets of application of image morphometry are (**Box 19.2**):

Linear distance measurement: Linear distance between two objects, such as cell-to-cell distance or basement membrane to cell distance, etc. is measured.

Object counting: It means to count the number of objects such as mitotic figures or any other particular object in a particular field area.

Form factor: This means measurement of area, diameter, perimeter and other geometric features of the object.

Area fraction: This indicates the measurement of particular areas in the total field area such as the measurement of AgNOR area occupied in the nucleus[1] or measurement of the area of reticulin fiber in trephine biopsy.[2]

BOX 19.2 Areas of application of image analysis

Morphometry
- Linear distance measurement: Distance between two objects
- Object counting: Number of objects in a field area is counted
- Form factor: Area, diameter, perimeter, etc.
- Area fraction: Relative area of the substance
- Mean nuclear volume: Nuclear volume from two-dimensional image
- Fractal dimension measurement
- Complex measurement: Cell-to-cell relation, roundness, etc.

Cytometry
- Relative DNA content
- Quantitative estimation of antigen

Chromatin pattern recognition

Complex measurement: With the help of image morphometry, complex measurements such as Cell-to-cell relation or measurement of roundness can be done.

Mean nuclear volume: Mean nuclear volume can be estimated in 2D planes with the help of sterotactic method.[3,4]

Fractal dimension: Fractal geometry is a relatively new area of the morphometry. Unlike Euclidean geometry, fractal geometry deals with non-integer dimensions.[5] The fractal dimension of an object describes the irregularity of the object. Measurement of fractal dimension may help in assessment of chromatin distribution, nuclear margin irregularity, tumor angiogenesis and collagen distribution in the section.[5] With the help of image morphometry, fractal dimension can be measured.

Nuclear morphometry may be used to do tumor grading, subtyping, diagnosis and prognosis.

Cytometry

In case of cytometry, the amount of dye within a certain tissue or cell or nuclei is measured with the help of image analysis. DNA cytometry measures the relative amount of DNA within the nucleus. The principal of DNA cytometry is that a DNA specific dye is used to bind with DNA stoichiometrically. The optical density of the Feulgen stained nucleus is directly proportional to the DNA content of the nucleus. So the optical density of the nucleus is measured to assess the relative amount of DNA. Finally, the optical density of the test nuclei is compared with the control nuclei. Commonly normal human peripheral lymphocytes are used for diploid control. For the successful DNA cytometry, two things are needed: (1) the nuclei should be total and (2) each nucleus should be discrete as overlapping nuclei may give wrong results. DNA image cytometry may help in identification of aneuploid tumor cells in effusion fluid or bladder wash cytology sample. Aneuploidy in these samples usually indicates malignancy. It can also be used for tumor grading on the basis of ploidy.[6]

Image cytometry is also used to measure the amount of immunohistochemically stained proteins in cells such as estrogen receptors or p53 antigens.[7] The amount of stained material in immunohistochemistry is not stoichiometric. So, stringent calibration of the immunohistochemistry staining is required for quantitative assessment of antigen.

Chromatin Pattern Recognition

With the help of image analyzer, it is possible to assess the chromatin pattern recognition of the nuclei. Recently fractal geometry is also used for chromatin pattern recognition.[8] The assessment of chromatin pattern may help to classify the different cells.

DIGITAL IMAGE ANALYSIS: CERTAIN PROBLEMS

Disease identification: By the help of visual microscopic examination, we reach to a diagnostic identification of the disease. In this identification process, we collect the information and assess the diagnosis with certainty. The adequate amount of information on a particular entity makes us more certain. In contrast, a classification

procedure operates in a closed feature space. The statistical classification procedure is dependent on a certain number of features to discriminate the members of different classes. A class member should have the typical values of the certain features. In an unknown entity, the values of only the features of interest are computed and according to the different values, the classification is made. Traditional image analysis may be efficient in classification, but not so much reliable for diagnostic identification.

Decision on individual patient: Till now the various analytic methods deal with the population as a whole. There is not much methodology on individual case based decision. Massive database of the patients for a particular diagnostic condition is needed to solve this issue. The exact clinical outcomes of the patients are also needed. Data mining technique with the help of artificial neural network (ANN) may be helpful which may trace patterns of mutual correlation among the variables in data sets.[9]

Selection of images of interest: Selection of images for interpretation is a challenge to the automated image analysis. A successful automated image analyzer should have the capability of scene processing, segmentation and feature extraction. Motironi et al.[10] applied a machine-based system to quantify the basal cell layer in histopathology sections of the prostate. This machine vision-based system allowed objective assessment of conventional diagnostic criteria and concepts which were not readily noted by the human perception.

Large amount of data handling: First and foremost, to automate pathology, it is essential to digitize slide data. For interpretation of any slide, large amount of data has to be captured and processed rapidly. This is still a challenge to the image analyzer.

Other technical challenges: There are other challenges of image analysis and interpretation such as rapid panning and zooming of the microscope, focusing to the point of interest, database storage for metadata, visualization and pattern recognition (**Box 19.3**).

BOX 19.3 Limitations and difficulties of image analysis

- Disease identification: Exact identification of disease difficult
- Decision on individual patient: Overall classification of cases possible but difficult to decide on individual cases
- Selection of images of interest: Objects of interest are difficult to identify by automated analysis
- Large amount of data handling is problematic
- Other technical challenges: Rapid panning, zooming, focusing, data storage

OTHER DEVELOPMENTS IN IMAGE ANALYSIS

Laser Scanning Cytometry

Laser scanning cytometry (LSC) is a relatively new area of image cytometry with significant advantages (**Box 19.4**) over the routine image cytometry.[11,12]

BOX 19.4 Laser scanning cytometry

Principle
- Fluorescent labeled cells on the smear are hit by laser beam
- Intensity of fluorescence measured
- Cell image is also stored for correlation

Advantages
- Rapid technique
- No single cell preparation or special processing
- Cell loss negligible so can be done on scanty cellular smear
- Cell can be stored for long time
- Morphologic correlation with fluorescence data possible

Applications
- DNA ploidy estimation
- Immunophenotyping
- Cell proliferation fraction
- Apoptotic cell counting
- Quantification of antigenic substance

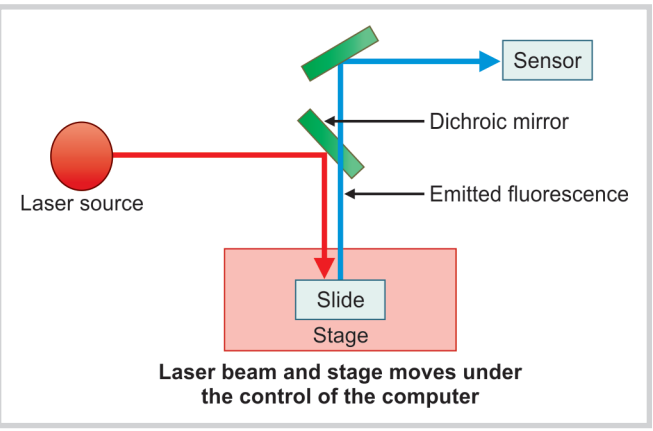

Fig. 19.5: Schematic diagram showing the basic principle of laser scanning cytometry

Principle (**Fig. 19.5**): In LSC, the specimen is fixed on a glass slide, and with the help of laser beam each cell is scanned automatically. The slide position and laser beam are moved automatically under the control of a computer. The cells are stained with antibody tagged with fluorescence dye. Fluorescent labeling does not need any enzyme amplification so the measurement of intensity of fluorescence helps in the accurate quantification of antigen in this fluorescence-based study. Laser excited fluorescence emitted, from flurochrome stained individual cells, is measured in multiple wavelength. A fluorescence value for each cell is recorded along with the X-Y position of the individual cells. So, it is possible to correlate directly the immunofluorescence measured with the cytomorphology on a cell by cell basis. The various other features of objects such as area, diameter, perimeter, etc. can also be measured simultaneously.

Advantages of LSC: There are various advantages of LSC:[13]
- This is a very *rapid technique* in comparison to simple image cytometry done by light microscope

- At LSC, the *cells are on a solid support* as they are placed on a glass slide. It is not necessary to have single cell or nuclei preparation as that is required for flow cytometry. Touch preparation or FNAC smears can also be used for LSC.
- There is *no need for* centrifugation or *special processing* for LSC so cell loss is negligible.
- The cells are permanently fixed on glass slides for studies in enzyme kinetics or other time consuming process and the measured cells can be relocated to correlate with visual examination.
- The analyzed cells can be stored for long periods.

Applications

There are various applications of LSC:

- With the help of LSC, the measurement of DNA content is possible and the ploidy status of the cell can be correlated with the morphology
- Immunophenotyping of cells
- Laser scanning cytometry is helpful in demonstration of apoptotic cells by staining of propidium iodide, Rh 123 or FITC tagged Annexin V
- Quantification of estrogen and progesterone receptors in breast carcinoma
- Quantification of actively proliferating cells with the help of Ki 67 immunostain.

Confocal Microscopy

Confocal microscopy is a remarkable development in the field of image analysis. In a conventional wide field light microscope, the entire specimen is illuminated by light and the image can be viewed directly. In contrast, the method of image formation in a confocal microscopy is fundamentally different. Illumination is achieved by scanning one or more focused beam of light, usually from a laser or arc-discharge source. The high intensity laser light is reflected from a dichroic mirror, i.e. the mirror which has the selected property to transmit and reflect the light of a particular wavelength and is brought to a point of focus by the objective lens at the labeled "plane of focus" (**Fig. 19.6**). Emitted fluorescence from the point of focus of the specimen passes back through the dichroic mirror and the confocal aperture to the photomultiplier detector. It is important to note that the fluorescence will also be emitted from planes above and below the plane of focus, but this light will be prevented from reaching the detector by the pinhole/confocal aperture. Ultimately, the computer attached to the photomultiplier tube detector helps in building up the entire image pixel by pixel. By having a confocal pinhole, the microscope is really efficient at rejecting out of focus fluorescent light. Also by scanning many thin sections of the sample, a very clear 3D image of the sample is formed. The microscope has good resolution both horizontally as well as vertically. The fixation of tissue is often not required for the study of confocal fluorescence imaging. It is now also possible to record images within and around living cells in various proportions including thick tissue slices.[14] In contrast to ordinary fluorescence, the rapid assessment of the spatial distribution of extracellular and intracellular macromolecules, including proteins and nucleic acids is possible with the help of confocal microscope.[15]

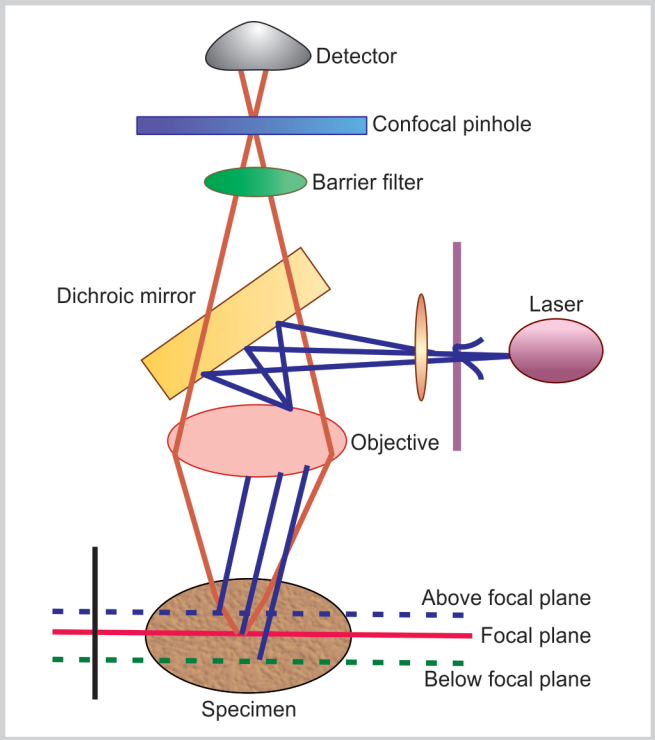

Fig. 19.6: Schematic diagram showing the basic principle of confocal microscopy

KNOWLEDGE-BASED EXPERT SYSTEM FOR DATA INTERPRETATION OF IMAGE ANALYSIS

Data analysis and interpretation is a very important aspect of image analysis. There is a major advancement in this area. Various computer-based expert systems are available now for the data interpretation. These systems are based on a Bayesian belief network (BBN), ANN and logistic regression analysis.[16-21]

Bayesian Belief Network

Bayesian belief network is a knowledge-based decision support system.[22,23] The network is comprised of multiple nodes that means multiple evidences or features with multiple outcomes. Such as for diagnosis of carcinoma of the breast, the various cytological features are the nodes. Each cytological feature may have various outcomes such as nuclear enlargement may be mild, moderate or marked. Therefore, the nuclear enlargement (node) has three outcomes (mild, moderate and severe). Belief in various outcomes is stored in each node and is expressed as a probability. Each node is connected with other by links. Links between the nodes are quantified by conditional probability (CP) matrix. When the descendant or evidence nodes is connected to the parent node or final outcome node, the CP matrix in between the node expresses the probability of getting a particular outcome. Such as in the diagnosis of breast carcinoma, the final outcome may be benign or malignant or in urine cytology, the outcome may be benign, atypia or malignant (**Fig. 19.7**).

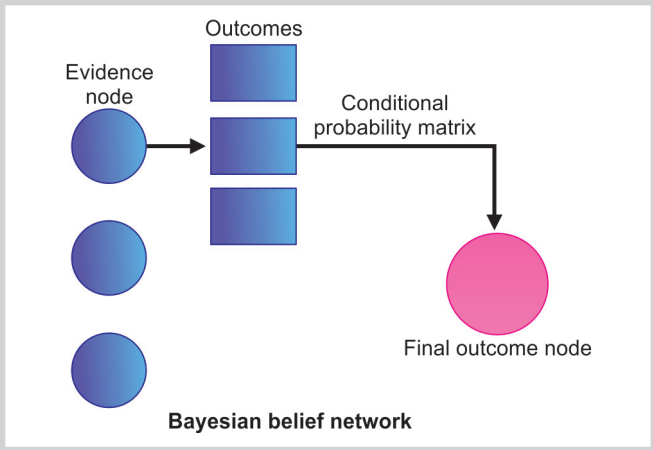

Fig. 19.7: Schematic diagram showing the basic principle of Bayesian belief network

Bayesian belief network has been applied in cytology for diagnosis and grading of carcinomas. It is a promising field expert based knowledge system.[17,24]

Artificial Neural Network

Artificial neural network is a powerful non-linear statistical classifier whose architecture is designed on the basis of the human brain (**Box 19.5**).[9]

How ANN Works

Artificial neural network (**Fig. 19.8**) is made up of many simple processing units, which are bundled together in a complex communication network. Each of this processing unit is known as "node" which simulates "neuron" of the brain. Each node receives signal from other nodes or from external stimuli. It then sends signals to other nodes via connection. The signal that a neuron receives from other neurons are weighted and then summed to produce an overall activity level in the neuron.

The layers of ANN consist of: (1) an input layer, (2) single or multiple hidden layers and (3) an output layer. The nodes of input layer receive input signals from the external stimuli. The output layer consists of nodes that communicate with the output of the system to the user or external environment. There are single to multiple hidden layers in between the input and output layers. When the input layer receives the input signal, its neuron produces an output signal. This becomes input to the other layers of the system. Ultimately after a certain situation, the output layer sends its signal to the user or external environment.[25] To provide training to ANN, three non-overlapping sets of data are used: (1) training set, (2) validation set and (3) test set. The training set is used for the adjustment of weights during training and validation set is used for maintaining a topological network. The results of the test set can be considered as a true prediction of ANN performance on new data.

BOX 19.5 **Artificial neural network**

Basis: ANN simulates human brain. Here, instead of neurons, there are multiple interconnecting nodes. Each node receives signal from other nodes or from external stimuli. Ultimately final outcome node sends the result

Layers
- Input layer
- Hidden layer
- Output layer

Training: Three sets of data
- Training set
- Validation set
- Test set

Advantages
- Robust and can tolerate missing data
- Can analyze nonlinear complex data
- Can take decision on individual cases
- Faster and can process parallel data
- Learn from example rather than logic

Applications
- In automated analysis of cases particularly in cervical cancer
- Analysis of complex data in gene microarray
- Diagnosis of difficult or complex cases

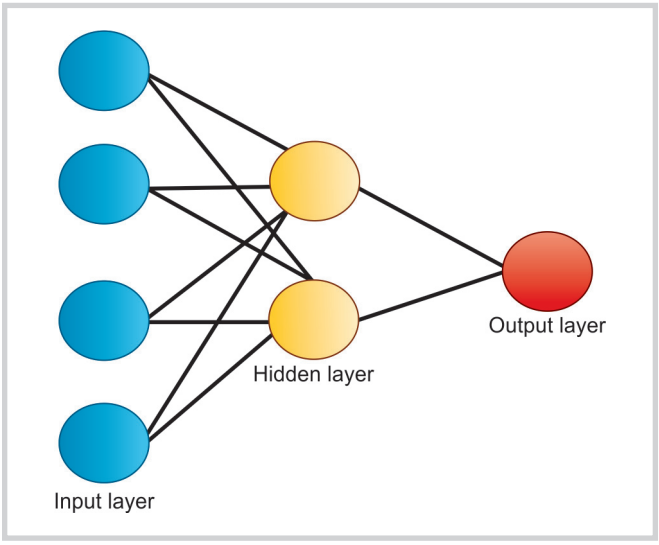

Fig. 19.8: Schematic diagram showing the basic principle of artificial neural network

Advantages and Disadvantages

Artificial neural network has certain advantages. It learns by example rather than the specific computer code for a classification procedure. ANN is robust and can tolerate missing data. There are certain problems in ANN implementation. ANN lacks transparency. There is no checking of information storage and processing in ANN. There is also ambiguity of the optimal architecture of ANN and the risk of overtraining of ANN cannot be ignored.

Application

In the field of cervical cytology screening, PAPNET system is commercially available which applies ANN for image analysis. Conventional Papanicolaou stained smears can be screened by PAPNET system and no special processing of the sample is needed. There are a number of studies on the role of PAPNET on re-screening of cervical smears. All these studies showed that these techniques helped to detect an increased number of false negative cases in comparison to manual screening.[26-28]

The potential value of morphometry and ANN for discriminating benign from malignant nuclei and lesions of the lower urinary tract on images of routinely processed voided urine smears were investigated by Karakitsos P et al.[29] ANN has been applied in the FNAC smears of breast carcinoma to differentiate ductal carcinoma from lobular carcinoma.[30] Recently, it was also applied in effusion cytology smears to identify malignant cells from mesothelial cells.[31] Application of ANN promised to become a powerful tool for everyday practice in the cytology laboratory.

CONCLUSION

In recent years, the cost of the computer has been reduced significantly with the simultaneous increased computing power. So image analysis is now within the reach of any good pathology laboratory. Newer technology, such as laser scanning cytometry, is very fast and it has overcome the major limitation of the traditional image analyzer. The confocal cytometry can measure the geometrical features of 3D cell clusters and can reconstruct the images in the computer screen. The expense and technical expertise required to master these tools, however, often present barriers to wide use of cytometry equipment. Improved algorithms, the ever-increasing processing power of computers and progress in biochemical specimen preparation techniques make it likely that eventually useful automated prescreening systems will become available and in the future, image analysis will take a major role in routine laboratory life.

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CHAPTER 20

Flow Cytometry

Chapter Contents

INTRODUCTION

Flow cytometry (FCM) is the study of different characteristics of a single cell in the fluid stream. This technology has developed rapidly in the past few decades. Unlike the older instrument, the modern flow cytometer is now smaller, user friendly, versatile and less costly. The instrument is now used not only for research, but also for routine clinical activities.[1-5]

PRINCIPLES OF FLOW CYTOMETRY

Single cells in fluid suspension are needed to do flow cytometry. These cells are stained with a fluorescent dye. The fluorescent dye binds with the specific components of the cell. DNA specific dye binds with DNA stoichiometrically and similarly antibody tagged with the fluorescent dye binds with the cell surface antigen or receptors. The flurochrome stained cells flow singly in front of a laser beam. When the laser beam of particular wavelength hits the cell then the cell absorbs light and emits fluorescence. In case of multiple flurochrome dyes, the excitation beam of light may be of same wavelength but the emission beam of light from different flurochrome dye will have different wavelengths. With the help of appropriate filter, the simultaneous evaluation of multiple colors (parameters) for each cell can be recorded. The flow cytometer receives the emitted light signal with the help of the photodetectors and then converts it into digital signals for storage on the computer. The intensity of this digital signal is graded on a relative scale known as channel. The results are then displayed as histogram or two-dimensional dot plots.

CYTOLOGY SAMPLES

Different types of cytology specimens can be processed for FCM:

1. Fine needle aspiration cytology materials: Lymph node, breast, lung, prostate, etc.
2. Exfoliative samples: Effusion fluid, cerebrospinal fluid (CSF), bladder wash

A cytology specimen has some special *advantages* over the histopathology tissue. These are:

- Cytology sample is easy to process and requires less disaggregation and thereby single cell preparation is easier to make for FCM
- It is relatively easy to procure cytology sample
- Multiple areas of the tumor or lymph nodes can be collected for processing
- As the cells are viable so functional studies can be done.

BOX 20.1 Citrate buffer

- 85.3 gm sucrose
- 11.8 gm trisodium citrate (Sigma)
- 50 mL of dimethyl sulfoxide in 100 mL of water pH adjusted at 7.6

Collection

The cytology samples can be collected in 1 mL of citrate buffer solution (**Box 20.1**). The sample can be processed immediately or may be kept at -70°C to process later on.

Control

Lymphocytes from the peripheral blood samples of healthy individuals may be kept as an external control for DNA FCM. Certain general requirements are needed for instrument control such as optics, fluidics and electronics. This is usually done with commercially available fluorescein-labeled beads. The individual laboratories should always follow the recommendations of the manufacturers of the instruments on a daily, weekly or monthly basis.

For flow cytometric immunophenotyping (FCI), both positive and negative controls should be used. Negative control is needed to eliminate background autofluorescence and nonspecific staining. Positive control is needed to verify the working condition of the reagents. The fresh normal blood sample is ideal for the control sample. Commercially available preparations may be more convenient as these preparations are stable and more reproducible.

Dyes Used

Propidium iodide, ethidium bromide, Hoechst 33342, diamidinophenylindole (DAPI), etc. is used for DNA content measurement. The presence and quantity of surface receptors or intracellular antigens are measured with fluorescein isothiocyanate (FITC), phycoerythrin (PE), asllophycocyanin (APC) and Texas-red (**Box 20.2**). Most of the flurochrome dyes are excited in same wavelength (488 nm) and they emit light of different wavelengths. RNA content is measured with acridine orange, pyronin Y and oxazine 1. Intracellular enzyme activity is detected with the help of chromogenic or fluorogenic substances.

SAMPLE PROCESSING

The aspirated material should be centrifuged and re-suspended in citrate buffer. The single cell suspension is done either mechanically by repeated syringing through nylon mesh or by trypsinzation.[4,6] The cell concentration in the buffer is kept as at least 2 x 10^6 cells/mL. The cells are permeabilized and stained with a DNA specific dye such as propidium iodide.[3,4] In authors' laboratory, they use modified Krishan's mixture (**Box 20.3**).

BOX 20.2 Dyes used for flow cytometry

DNA content measurement
- Propidium iodide, ethidium bromide, Hoechst 33342, diamidinophenylindole (DAPI), mithramycin, chromomycin

RNA content measurement
- Acridine orange, thiazole orange, pyronin Y, thioflavin T and oxazine 1

Surface receptors or intracellular antigens
- Fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin-chlorophyll-protein and allophycocyanin(APC), Texas red

BOX 20.3 Modified Krishan's buffer

In 250 mL of distilled water add:
- 0.25 gm sodium citrate
- 0.005 gm RNAse (Sigma, catalog no R-4875)
- 0.75 mL NP-40 (Sigma, catalog no N-6507)
- 0.0125 gm Propidium iodide (Sigma, catalog no P-4170)

The pH of the buffer maintained at neutral pH before adding Propidium iodide

The DNA stain is done by adding modified Krishan's mixture (1 mL) with 1 mL cell sample.[7]

In case of FCI, the samples are washed in phosphate buffered solution (PBS) and then re-suspended and kept at 1.5 × 10^6 cells/mL into multiple small aliquots. In each aliquot, antibody tagged with fluorescein dye is added and incubated for 15 minutes. RBC is lysed by adding lysing solution. The specimen is then centrifuged, supernatant solution is discarded and the cells are fixed in fixative solution and FCI is performed.[8]

TARGET OF APPLICATIONS

Flow cytometry can be used for quantitative measurement of various cellular characteristics (**Box 20.4**). Many of the targets of application of FCM are research oriented.

Most common applications of FCM in clinical laboratory are immunophenotyping, DNA content analysis and cell cycle analysis (**Box 20.5**). In addition, FCM is used also for histocompatibility cross matching, HLA-B27 detection, CD 4 and CD 8 counting for immunodeficiency, reticulocyte enumeration and paroxysmal nocturnal hemoglobinuria detection.

DNA Content and Ploidy Analysis

DNA content measurement is based on the stoichiometric binding of the DNA-specific dye with the DNA of the nucleus and subsequent measurement of emitted fluorescence from the dye-DNA complex. The emitted fluorescence is supposed to be proportional to the DNA content of the cells and is represented as channel number which as mentioned before is an arbitrary

BOX 20.4 Measurement of cellular features

- Cell size
- Cell shape
- DNA content and cell cycle analysis
- RNA content
- Mitochondrial function
- Cellular protein, lipid, hemoglobin, calcium
- Intracellular and surface receptors
- Apoptotic cells

BOX 20.5 Common clinical applications of flow cytometry

- DNA ploidy analysis by measuring DNA content
- Cell cycle analysis by S phase estimation and Ki67 measurement
- Immunophenotyping of lymphoma and leukemia: by various CD markers
- Histocompatibility cross matching: by IgG and IgM antibody
- HLA-B27 detection
- CD4 and CD8 counting for immunodeficiency
- Reticulocyte enumeration
- PNH detection: by CD55 and CD59 estimation

value depending on the machine's initial set up. The data are displayed as DNA histogram. As the majority of the normal cells contain diploid (2n) chromosome, so they emit a certain amount of fluorescence represented by a channel number. This forms a single peak known as the diploid peak in DNA histogram (**Fig. 20.1**). As the cells in G_2M phase contain the double amount of DNA (4n), so they emit the double amount of fluorescence and are present in double channel number in the DNA histogram forming a small tetraploid peak. In between these two peaks, the cells have varying amount of DNA from 2n to 4n and they are in synthetic (S) phase. Any peak, other than these two peaks should be considered as an aneuploid peak.

Following information is obtained from DNA FCM:

- Identification of aneuploid cell population
- DNA index
- Proliferative cell fraction (% of S phase)

DNA ploidy: During proliferation of the cells in malignant tumor, there may be a generation of clone of malignant cells that contain a specific amount of DNA other than diploid DNA content. This clone of cells containing the abnormal amount of DNA is known as aneuploid cells. In DNA histogram, the aneuploid population will form a peak somewhere other than diploid peak.

The relative DNA content of the aneuploid population of cells is calculated by DNA index.

$$\text{DNA index} = \frac{\text{Mean channel number of aneuploid peak}}{\text{Mean channel number of normal diploid peak}}$$

The different types of aneuploidy are:

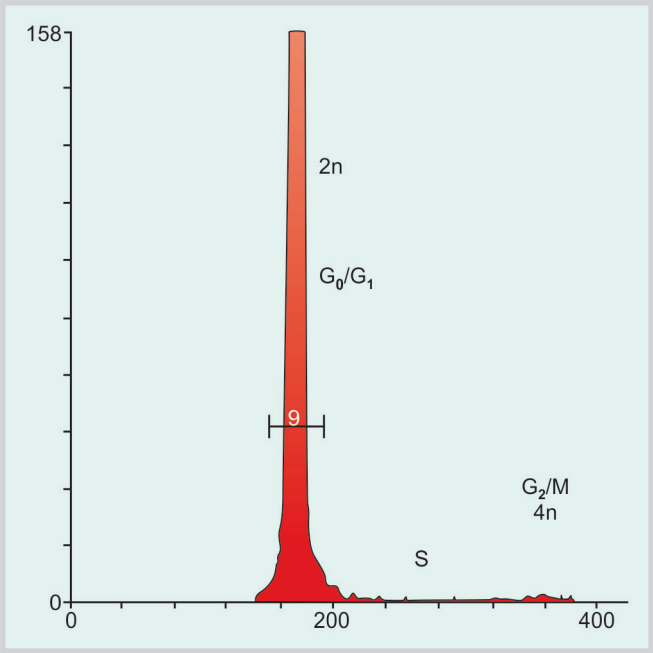


Fig. 20.1: Diploid DNA peak in DNA flow cytometry

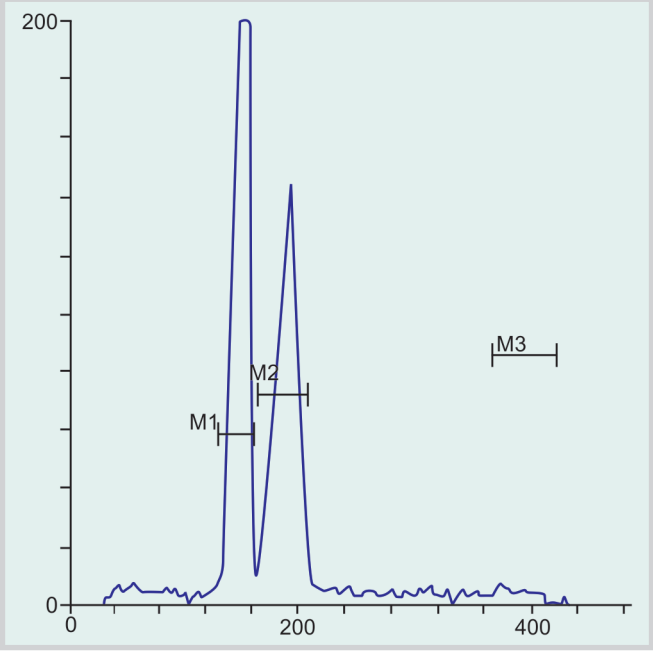


Fig. 20.2: DNA histogram showing hyperdiploid aneuploidy

Hyperdiploid aneuploidy (**Fig. 20.2**): If the aneuplod peak is in between diploid or tetraploid (DNA index more than 1 and less than 2).

Hypodiploid anuploidy: Aneuploid cell population contains less than the 2n amount of DNA and form a peak left of the diploid peak (DNA index less than 1).

Tetraploid aneuploid: Aneuploid population forms a peak that overlaps with G_2M or tetraploid peak. This is difficult to distinguish from G_2M cells. However, the aneuploid G_2M peak contains more than 20 % cell population (DNA index is 2).

Hypertetraploid aneuploidy: Aneuploid population of cells contains more than 4n amount of DNA and form a peak right to the G_2M peak (DNA index more than 2).

S phase: S phase fraction of cells is calculated as the population of cells in between G_0G_1 and G_2M peak. This is easily calculated from the DNA histogram.

Clinical Relevance

DNA FCM is considered as useful for diagnosis and prognosis of tumors. DNA aneuploidy and high S phase (proliferative fraction) percentage is commonly noted in malignant tumors and may be used for diagnosis of malignancies.[3-5] However, false negative or positive cases may occur because many malignant tumors are diploid and rare benign tumor may show DNA aneuploidy.[9]

Diagnosis

Effusions

There is a variable success rate of DNA FCM in the diagnosis of malignancy in effusion fluid. The false negative rate of FCM is as high as 41% and false positive rate of FCM is only 2–3%FCM.[10,11] DNA FCM alone is not cost effective and dual color FCM is more helpful in diagnosis of malignancy.[12]

Bladder Washings

DNA FCM of bladder wash specimen is a helpful ancillary technique for detection of malignancy of the bladder. DNA FCM shows 78–93% sensitivity.[5,13-15] DNA FCM is especially helpful in follow-up cases of urothelial carcinoma of the bladder. DNA ploidy estimation of gated population of cytokeratin positive cell is more helpful to detect malignancy in urine.[16]

Cerebrospinal Fluid

DNA FCM is not very helpful in the detection of malignancy in CSF because of low cellularity in CSF. However, combined use immunophenotyping and DNA FCM may be useful in the diagnosis of lymphoma in CSF.[17]

Prognosis

It is considered that DNA aneuploidy and high S phase is a poor prognostic factor in solid tumors, such as bladder, prostate, ovarian and endometrial carcinomas.[18-20]

Immunophenotyping

Presently, FCI is widely used in the detection of various antigen or receptor markers on the surface of the cells. Cluster of differentiation (CD) indicates the specific receptor or ligand on the cell surface. More than 300 CDs are listed till now.[21] Similarly, a large number of monoclonal antibodies against various CD markers are also commercially available for FCM. Demonstration of various CD markers on the lymphoid cell surface has great impact in the subclassification of lymphomas and leukemia.

Diagnosis

Flow cytometry can demonstrate kappa and lambda chain on the cell surface of lymphoid cells. As non-Hodgkin's lymphoma (NHL) is monoclonal in origin, so in B-NHL, the neoplastic cells show light chain restriction and they show either lambda or kappa light chains. Therefore, the presence of light chain restriction indicates lymphoma. In FCM, the kappa/lambda ratio more than 4:1 or more than 1:2 is usually considered as an evidence of monoclonality. It is difficult to determine clonality of the T cell NHL. Predominant expression of CD4 or CD8 expression may be considered as surrogate markers of clonality of T-NHL. However, the aberrant expression of CD2, CD5 and CD7 expression may suggest T-NHL.[22] There is no doubt that the only way to diagnose T cell clonality is by demonstration of T cell receptor gene arrangement of molecular methods.

Subclassification

B cell NHL shows a specific pattern of CD marker expression on their surface and the use of a panel of markers along with cytomorphology is very helpful in subclassification of NHL (**Figs 20.3** and **20.4**).[22] This has been discussed in detail previously (*see* Chapter 17, page 237). In fact, FCM is especially useful for lymphoma phenotyping of FNAC material as the cells are discrete. FCM has certain unique *advantages* over routine immunocytochemistry (**Table 20.1**). These are:

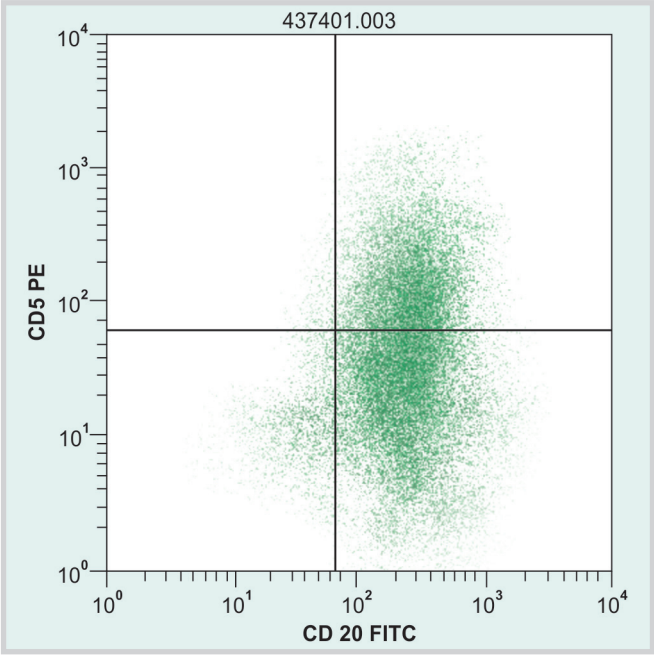
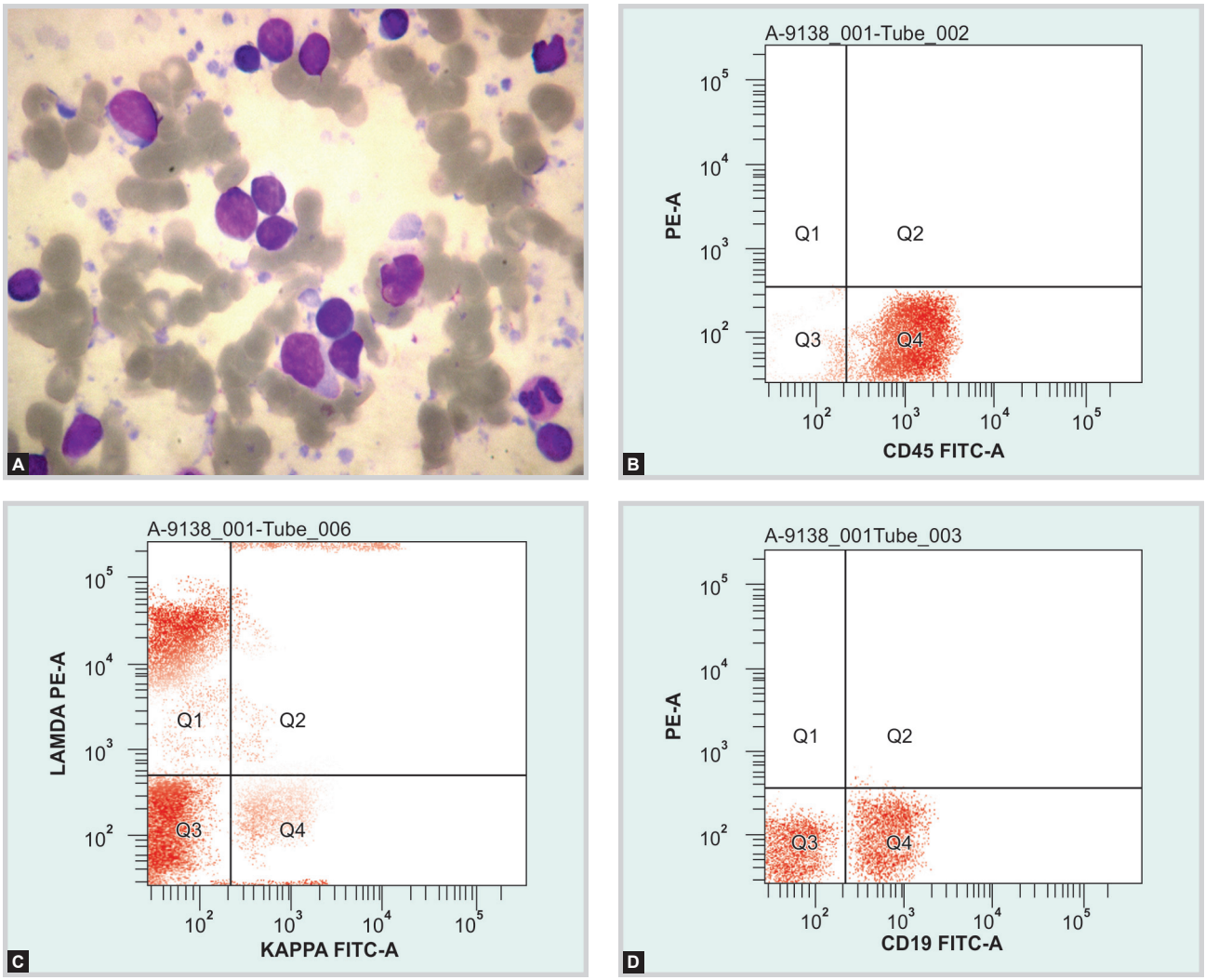


Fig. 20.3: Both CD5 and CD20 positivity in a case of small lymphocytic lymphoma



Figs 20.4A to D: (A) Fine needle aspiration cytology smear of an orbital lymphoma showing discrete lymphoid cells. Individual cells show round nuclei and scanty cytoplasm. Background of the smear shows lymphoglandular bodies (May-Grünwald-Giemsa stain X HP); (B) Predominant cells are CD45 positive; (C) Cells show predominantly lambda light chain expression indicating monoclonality of the cells; (D) Majority of the cells are CD19 positive indicating B cell origin

TABLE 20.1: Comparison of FCM immunophenotyping and immunocytochemistry

	FCM Immunophenotyping	*Immunocytochemistry*
Processing speed	Very rapid	Slow
Subjectivity	Objective	Interpretation is more or less subjective
Antibody panel	Wide variety of CD markers can be used	Limited CD markers are used in cytology material
Dual expression of CD markers	Possible	Not feasible
Additional features	Possible to estimate multiple other features	Not feasible
Quantitation of CD positive cells	Possible and easy	Laborious process to estimate manually
Quantitation of intensity of antigen positivity	Possible and easy to do	Not possible
Morphology of the cells	Not possible to visualize	Possible to visualize
Archival preservation	Not possible	Possible to preserve for a long time

Abbreviations: FCM, Flow cytometry; CD, Cluster of differentiation

- It is a relatively fast technique and large number of cells can be analyzed rapidly
- A large panel of CD markers can be used in the FCM
- Dual expression of CD markers can be demonstrated
- Many other additional features such as DNA ploidy, cell cycle analysis, etc. can be done simultaneously along with immunophenotyping
- Flow cytometry is helpful to measure the percentage of positive cells along with the intensity of antigen expression in individual cells

LIMITATIONS OF FCI

Flow cytometric immunophenotyping has certain limitations (**Box 20.6**):

- Inability to correlate morphological findings with the FCI data is one of the major defects of FCI. Newer technology such as LSC may overcome this problem
- Admixture of benign reactive components of the lymph node such as vascular or stromal material
- Inability to identify Hodgkin's lymphoma because of scanty R-S cells in the specimen
- Necrosis, blood mixed sample etc. may produce faulty reporting and gated population of lymphoid cells are needed
- All cases of B-NHL may not always show light chain restriction. This may be due to failed expression of light chain on the surface of the cell
- Flow cytometry is overall a costly technique and needs good skill.

USES OF FCI

Apoptosis

Flow cytometry is one of the important technology to detect apoptotic cell death because the apoptotic cell death can be assessed with the help of light scatter, plasma membrane changes, and DNA content. Apoptotic cell shrinks and produces a loss in forward light scatter (FSC).

Flow Cytometry in HIV infection

Monitoring of CD4 and CD8 count in the blood sample of HIV infected patients is a mainstay of clinical flow cytometry. During the progression of HIV, the blood CD4 count decreases, therefore, the measurement of absolute CD4 count provides a valuable tool for monitoring disease progression or treatment response to HIV infected patients.

BOX 20.6 **Limitations of flow cytometric immunophenotyping**

- No morphologic correlation
- Admixture of benign cells
- Costly and sophisticated technique
- Inability to identify HL
- No evidence of light chain restriction in certain number of cases

Reticulocyte Count

Flow cytometry analysis of reticulocyte is a standard and preferred method. Auramine O and thiazole orange are FDA proved reagents for reticulocyte analysis using FCM. Reticulocyte count by FCM is precise and sensitive over the manual method.

Predicting Response to Monoclonal Therapy

Monoclonal antibodies are targeted against the specific receptors on cancer cells. Her-2 is a growth receptor that is present in the cells of the breast cancer patients. Monoclonal antibody Trastuzumab is developed against the extracellular domain of Her-2 for treatment of breast cancer.[23] Presently, monoclonal antibodies are used for treatment of non-Hodgkin's lymphoma. Rituximab, a monoclonal antibody directed against CD20 on B cells, is used in follicular or low grade NHL.[24] Similarly anti-CD33 is used against myeloid cells and anti-CD52 is used against lymphoid cells. The measurement of monoclonal antibody CD20 with the help of FCM is helpful in follow-up of cases after rituximab therapy (anti-CD20 antibody).[25]

Detection of Minimal Residual Disease

Flow cytometry is also very useful for the detection of leukemia or lymphoma associated immunophenotype of the neoplastic cells for the assessment of minimal residual disease. It is time saving and cost-effective technique. It has been shown that FCM is almost equally effective as quantitative PCR in the detection of minimal residual disease.[26]

Soluble Antigen Assessment for Transplant Patient Monitoring

OKT3 is a mouse anti-human antibody and is used to treat transplant rejection patient. Circulating concentrations of OKT3 can be quantified FCM that helps in disease monitoring.[27]

FUTURE OF FLOW CYTOMETRY

The FCM instrument is developed significantly in last few decades and there are also vast numbers of monoclonal antibodies commercially available for FCM. Therefore, multiple characteristics of single cells can be assessed by single cells. Presently, FCM is used in clinical laboratories for DNA content measurement, cell cycle assessment and immunophenotyping of lymphomas/leukemias, reticulocyte count, HIV patient monitoring, and follow-up of monoclonal antibody based therapy. In future, FCM will be an integral part of routine laboratory techniques for diagnosis, and disease monitoring.

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CHAPTER 21

Liquid-based Cytology and Automation

Chapter Contents

INTRODUCTION

Computer science has progressed remarkably in the last few decades and like every other field, it has also a significant impact on cervical cytology. Automated devices have now been applied in the field of cervical cytology with the intention to have a more efficient system in smear preparation, recognition and interpretation of the slide.

The reduction of false negative cytology is the major aim of automation. There may be two types of error in a case of false negative cytology:

A. Sampling error: This means that the lesion is present in the body but is not properly sampled. Sampling error is one of the major sources of error.

Remedies: It is not entirely possible to avoid the sampling error, but with the help of liquid-based cytology (LBC) we can get the maximum number of cells on the smear.

B. Screening errors: Screening error may be due to failure to find out the representative cells on the smear or it may be due to interpretation error.

a. Recognition errors: It is just like to find out the "needle in the haystack". The representative cells may be too few to find out even after a laborious search or they may be obscured within the blood, mucus or inflammatory cells and necrotic debris. Cytoscreener may overlook these cells due to fatigue or due to the above mentioned technical difficulties.
   Remedies: (1) Liquid-based cytology (LBC) may provide a clean background of the smear which is free of mucus or blood. Moreover the proportionate amounts of cells are concentrated in a small part of the slide as a monolayer form. This may help to find out the abnormal cell more easily.
   (2) The automated adjunctive technique helps the manual screening by locating the path of the screening on the computer screen. Automated screening may also help to find out the abnormal cells.
b. Interpretation errors: Another possible source of errors is nonrecognition of abnormal cells. This may be because of professional incompetence or inadequate training.
   Remedies: Automated screening devices may initially help to find out the abnormal cells. However, trained cytopathologist is needed for further confirmation of the abnormal cells detected by automated machines.

AUTOMATION IN SPECIMEN COLLECTION AND PREPARATION

Liquid-based Cytology

For last few decades, cervical cancer screening is based on conventional preparation (CP) of cervical smear. Conventional cervical smear is a quick, relatively cheap technique and so far it has helped to reduce the incidence of cervical cancer significantly in many developed countries. However, the false negative rate of conventional cervical smear is as high as 29% and sensitivity

of this test varies from 47% to 62%.[1] Samples taken by CP may often be inadequate, nonrepresentative and may be obscured by blood, debris or may show air drying artifact. LBC technique is an important step in the preparation of cervical smear. It has overcome many limitations of conventional cervical cytology. Presently, LBC is widely accepted in many laboratories despite the high cost of this technique.[2-4]

Collection Procedure of LBC

The cervical sample is obtained using a combination of plastic spatula and cervical broom type device in the same manner of conventional screening. Alternatively, sample is collected by cervix brush supplied by the company. The sample is then rinsed into a vial containing preservative solution. The head of the device is kept in the vial containing preservative solution and the handle is removed. The sample is sent to the cytology laboratory for further processing. The cells are made discrete and a monolayer preparation is prepared.

Different Company Products

Presently, only two commercially available liquid-based technologies are approved by FDA, USA: ThinPrep Pap test and SurePath. ThinPrep was approved in 1996 and SurePath in 1999. The preparation techniques of the ThinPrep Pap test and SurePath has been compared in **Table 21.1**.

ThinPrep (*Cytic, UK*): Here, the cells are collected by a plastic spatula or cytobrush and then transferred to the transport media provided by the company. The vial containing the material along with the filter is introduced into the instrument. The filter rotates within the vial which contains the cells in liquid. The cells are dispersed mechanically by rotation of the filter. The negative pressure is used to draw the fluid through a filter made of neutral polycarbonate. The flow of fluid through the filter is constantly monitored to get an optimal quantity of cells. The cells trapped in the filter surface are then automatically transferred to a glass slide and fixed immediately (**Fig. 21.1**).

SurePath test (*AutoCyt Prep, Cytorich Prep*): The cells are collected with the help of plastic device and transferred to a transport fluid. In the laboratory, the vial is vortexed and strained to break up mucus and large cell groups and the cell suspension is treated through a density gradient centrifugation process to remove the blood and other insignificant material. The cell pellet is re-suspended and is then allowed to sediment onto a glass slide (**Figs 21.2** to **21.4**).

TABLE 21.1: Comparison of Pap test and SurePath techniques

Features	*ThinPrep Pap test*	*SurePath*
Collection	Preserve cyte fluid, methanol based	Cytorich fluid, Ethanol based
Single cell preparation	By rotatotry filter within the vial	By vortexing
Cell concentration	By negative suction	By density gradient
Monolayered cells	Present, good	No monolayered cells
Cell concentration	Relatively less	Good cell concentration

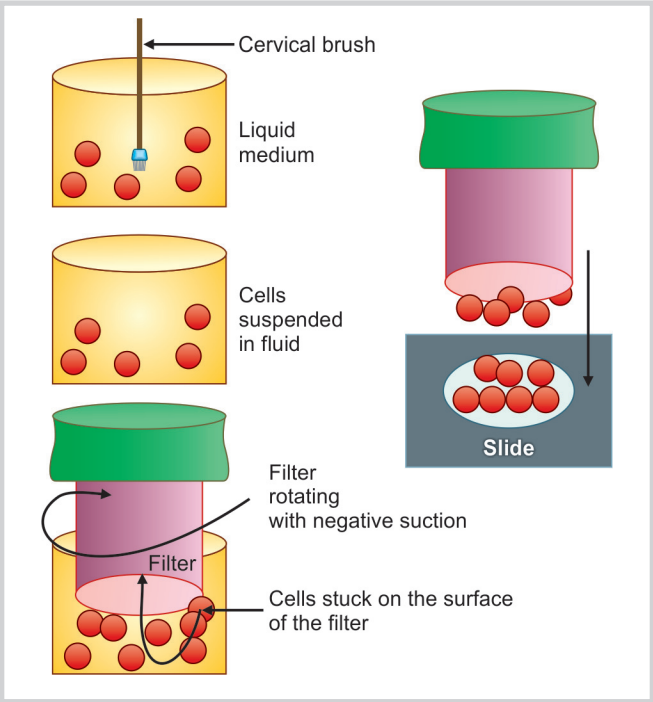


Fig. 21.1: Schematic diagram showing the basic principle of ThinPrep technique

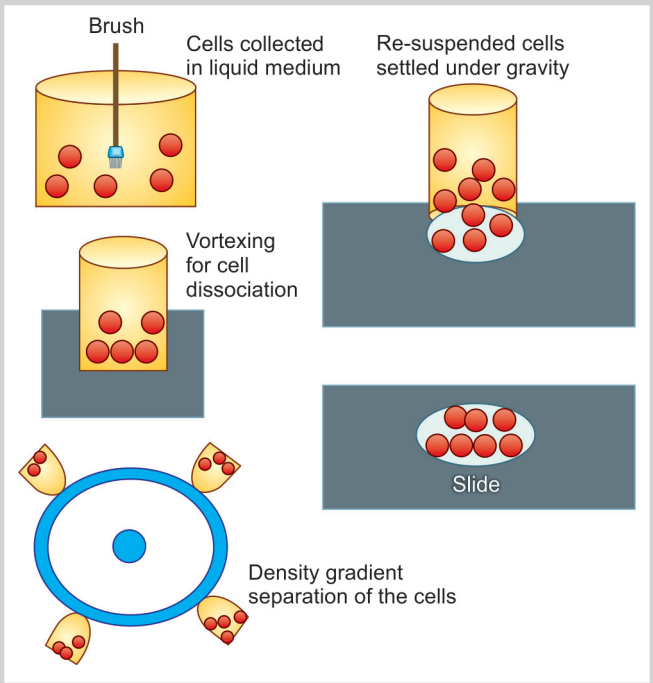


Fig. 21.2: Schematic diagram showing the basic principle of SurePath technique

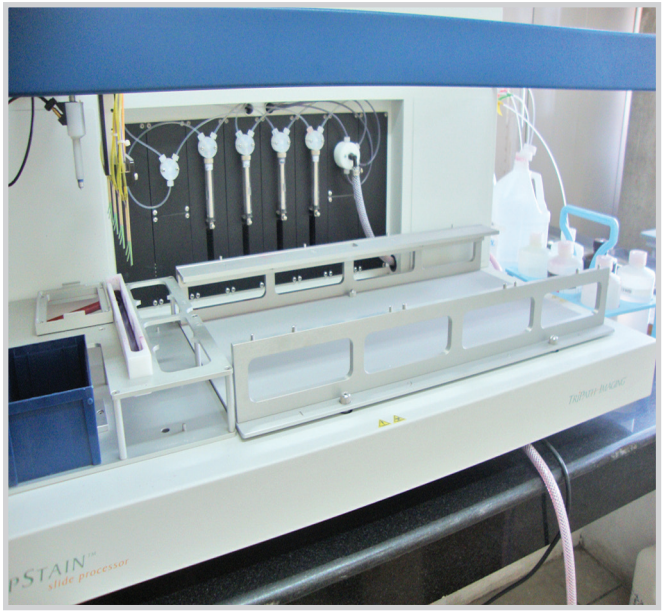


Fig. 21.3: Photograph of SurePath machine in our laboratory in PGIMER, Chandigarh, India

Fig. 21.4: Photograph showing preliminary preparation of sample in SurePath technique

Advantages and Disadvantages of LBC Over Conventional Pap Smears

Both LBC and conventional smear have certain advantages and disadvantages (**Table 21.2**). The main advantages of LBC are adequate representative material, complete avoidance of air drying, monolayered preparation and clean background (**Figs 21.5** and **21.6**). The background is clean from necrotic debris in LBC smear. However, it is still possible to recognize the tumor diathesis in case of squamous cell carcinoma of cervix. Infective organisms can also be identified in LBC smear. In fact, adequate training is needed for proper interpretation of LBC smear. One major advantage of LBC is that the residual sample can be kept for human papilloma virus (HPV) testing or any other ancillary tests. Moreover, the monolayered cellular smear is of great help in studying individual cells for automation.

Liquid-based cytology instrument is very costly. The overall processing cost of LBC is also much more than conventional preparation. As mentioned before, the laboratory technical staff also needs adequate training for interpreting LBC smear.

TABLE 21.2: Comparison of LBC and conventional preparation

	LBC	*Conventional preparation*
Sample collection	Liquid preservative	On glass slide
Air drying	No	Yes, at times
Number of cells	Much more and adequate	Less
Screening time	Less	More
Background	Clean	May be obscured by blood, polymorphs or mucus
Sensitivity	High	Low
Further ancillary test	Possible	Not possible
Cost of equipment	High	No cost
Cost to the patient	High	Low
Training	Needs adequate training	Routinely trained technician
Monolayer	Yes, in ThinPrep	No
Automation	Easy to implement	Difficult

Comparison of Interpretation

A large number of studies compared CP and LBC on cervical smear.[3,5-9]

Most of these studies claim that LBC reduces the inadequacy rate of cervical smear and it also increases sensitivity and specificity of the cervical smear compared to CP. However, these studies are mostly based on split sample collection procedure. At first, CP was taken and it was followed by procurement of a sample for LBC. Moreover, most of the studies were not randomized control trial. A large meta-analysis study by Davey E et al.[10] showed that LBC neither reduces the proportion of unsatisfactory slides nor it detects more high-grade lesions compared to CP. The authors refused to support the claim of better performance of

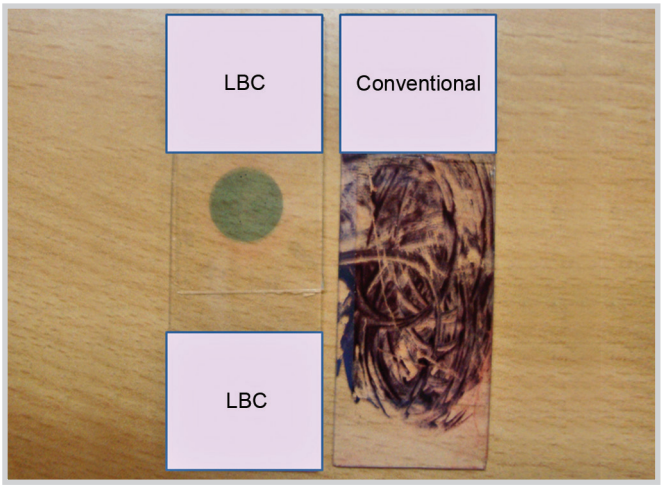
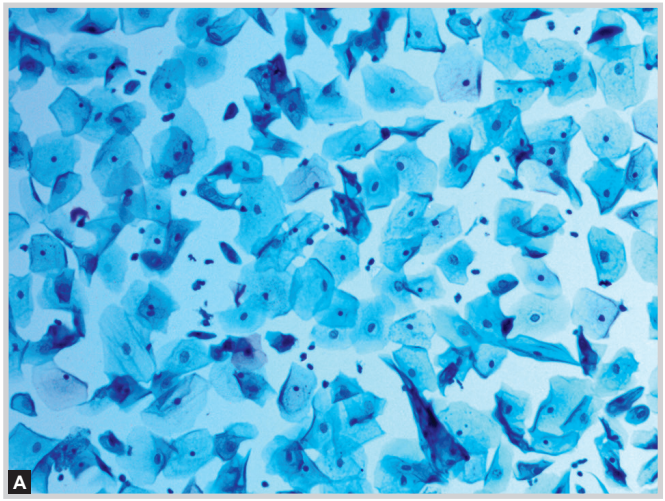
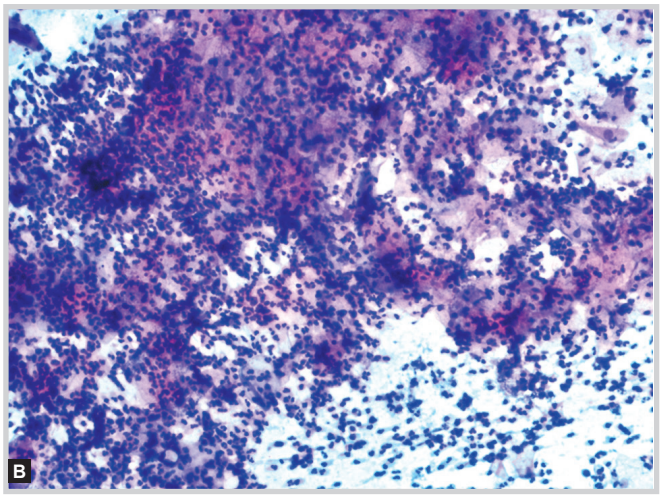


Fig. 21.5: Gross smears of liquid-based cytology preparation (SurePath) and conventional preparation





Figs 21.6A and B: (A) Clean background of liquid-based cytology preparation (SurePath) (Papanicolaou's stain X MP); (B) Dirty background and cell clumping of cells in conventional smear preparation (Papanicolaou's stain X MP)

LBC. Ronco G et al. performed a large randomized controlled trial study to compare CP and LBC.[11] They studied 22,466 patients in CP group and 22,708 patients in LBC group. HPV tests were also done in all these cases and colposcopy was performed in cytologically abnormal cases or HPV positive cases. A biopsy was taken from the abnormal area. They concluded that LBC does not show any statistically significant difference in sensitivity to conventional cytology for detection of cervical intraepithelial neoplasia of grade 2 or more. Moreover, LBC had lower positive predictive value as it detected more positive cases. However, the authors agreed that there was a large reduction in unsatisfactory smears.

ADJUNCTIVE DEVICES TO MANUAL SCREENING

Pathfinder

It is an additional device for manual screening. The system is attached to the stage of the microscope and traces the screening pathway of the smear and records the time taken by the cytotechnologist to screen each smear. The pathfinder is now obsolete and out of the market.

Trac Cell 2000 System

Trac cell 2000 system (Accumed) is a fully automated prescreening system that supports the screener. This system guides the screener about the areas of the slides to be screened. It locates the areas that need to be reviewed and also maps the pathway of the slide during screening. It also adjusts the speed, calculates the optimal focal plane and also the speed of the screening. The company claims that Trac cell 2000 system increases the productivity of the screener and also contributes strict vigilance over the cytoscreener.

AUTOMATED SCREENING

Automated screening procedure should be designed in such a way that no dysplastic or malignant cells should be passed as normal or negative and it should not label the normal cell as malignant cells. In addition, the automated screening technique should be economically cost-effective and it should shorten the period of screening. Therefore, the two main goals of automated screening are to increase the accuracy and productivity of the cervical screening.

Automated Screening Devices

Till date, there is no fully automated system available in the market. Presently, only two semi-automated systems are available BD FocalPoint GS Imaging System (previously known as Tri-Path AutoPap system) and HOLOGIC ThinPrep Imaging System (formerly known as Cytyc ThinPrep Imaging System). However, it is worthwhile to describe the first two FDA approved technologies, i.e. PAPNET and AutoPap 300 QC system for historical and learning aspects.

Redundant Systems

PAPNET

PAPNET system was designed and introduced by neuromedical system (Suffern, NY, USA). PAPNET was approved by FDA in 1995.[12] Later on, in the year 1999, the company was declared as bankrupt. Presently, PAPNET is out of the market.

The PAPNET system applied artificial neural network for the detection of abnormal cells. The PAPNET system consisted of two parts: (1) scanning station and (2) review station.

Scanning station: The cervical smears were sent to the central scanning station for screening. The scanning system is composed of an automated microscope system that is electronically programmed to scan the smear and capture cell images for computer processing. The smears were scanned by the microscope with attached electronic camera in different magnifications. The duties of the scanning station were to identify and record the potential abnormal cells with the help of artificial neural network. The images of the abnormal areas were stored digitally and sent to the review station for final interpretation.

PAPNET review station: The stored images along with the slides were sent to the referral laboratory for further decision. The images of the slides were examined on the computer screen and simultaneously the slides were reviewed under the microscope for final confirmation.

AutoPap 300 QC System

The AutoPap system was first introduced by Neopath in the year 1995 and was approved by FDA. Presently, this system is no longer available. This was an independent device, which automatically scans slides using image analysis algorithms. The AutoPap system generated an evaluation score ranging in value from 0 to 1.0 for each slide depending on the probability of abnormality of the cells in the smear. The higher the evaluation score, the more likely that abnormal cell is present.

Algorithmic Architecture of AutoPap System

The images are analyzed by five algorithms: (1) Stripe detection algorithm to detect dirt or glass scratches, (2) focus check algorithm to assume that the object is optimally focused, (3) single cell algorithm which classifies the cell and assesses the quality of the stain, (4) group algorithm for classification of the group, and (5) thick group algorithm for classification of thick groups, of cells. All the information is collected and finally the score is made to assess the likelihood of abnormality of each object.

Available Automated Screening Devices

BD FocalPoint GS imaging system (FPGS): BD FPGS is FDA approved automated device. This system includes BD FocalPoint™ Slide Profiler and the BD FocalPoint GS review station. BD FocalPoint™ Slide Profiler is a computer based automated screening devices that scans the slide in low and high magnification. More than 100 object analysis features are included in the screening algorithm of the system. The images are interpreted by an image analyzer software program and each slide is given a "device score" according to the probability of abnormality. Subsequently, the slide processing data is transferred to the review station. The system always categorizes the slide into no further review and review group.

BD FocalPoint GS review station: Here, the slides "for review" group are reviewed by the cytotechnologists. The slides for specimen adequacy are also reviewed. If no abnormality is detected by manual screening then the slides are labeled as normal and can be kept in archives. If any abnormality is detected then the slides are reviewed further by cytopathologists for final interpretation.

HOLOGIC ThinPrep imaging system: This system includes ThinPrep imaging system and ThinPrep review system.

ThinPrep image processor: Here the system acquires and process data from the ThinPrep test slides. This computer based system scans slide to find out the nuclei that are the largest and darkest as these nuclei are considered as abnormal. The imaging system always excludes the overlapping cells or the objects other than nuclei. The system screens the slide and records the locations of 22 microscopic fields that contain the potential abnormal cells.

Review scope: Review scope is an automated microscope with a motorized stage. It guides the cytotechnologist to review the 22 microscopic field of interest. If there are no abnormal cells in the 22 field of views then the slides are passed as normal. However, if there is any abnormal cell present then the whole slide is screened thoroughly.

Comparison of Manual and Automated Devices

Several studies claimed that automated screening done by either of these two techniques is more sensitive than routine manual screening.[13-16] However, these studies were not randomized and done in a small number of patients. Moreover, the studies are often sponsored by the company itself. In a large randomized control study,[17] the automation-assisted reading of cervical cytology was compared with manual report of cervical cytology smear considering histology as an end point. Both BD FocalPoint GS imaging system and ThinPrep imaging system were applied in two groups of patients and in both groups manual screening were also done. It was noted that automation-assisted reading was 8% less sensitive compared to manual report. Cervical smear categorized as "no further review" by automated screening was very reliable. Kitchener HC et al.[17] also noted that automated screening and manual screening were almost similar in cost-effectiveness issue. However, there was 60–80% increase in productivity for automated screening. It was concluded that there is no justification to introduce an automated screening technique in relation to sensitivity and cost effectiveness of cervical screening.

Problems of Implementing Automation

There are certain problems in automated screening devices.

Technical

- Data: Present generation computer technology can handle large amount of data. However, still it is difficult for a computer to handle the immense amount of data gathered during the screening of the individual slides.
- Autofocus: Continuous focusing of the cells, particularly overlapping cells, is a challenge.

Diagnostic

It is still very difficult for an automated system to make individual decisions in single cases.

Financial

Automated systems are commercially available but they are very costly for routine laboratories for daily use. This is particularly true for developing countries.

Medicolegal

It is still not settled about the medicolegal issues related to the machine given report in primary screening.

Automation: Advantages and Disadvantages

Automation of cervical smears has several advantages, such as higher number of false negative detection rate, no fatigability, consistency and relatively decreased turnaround time (**Table 21.3**).

TABLE 21.3: Comparison of the automated screening versus manual screening

	Automated screening	*Manual screening*
Fatigue	The machine does not feel fatigue	A man may feel fatigue
Logic	The device follows the standard direction of logic consistently	A man may not be uniform in approach
Time	The machine takes a fixed time and usually shorter period	Man takes longer time to screen
Interpretation	The machine cannot take final interpretation	Man can take meaningful interpretation
Training	The machine does not need training	Man needs training.
Cost	The machine is very costly	Manual screening is less costly

However, it is important to note that automated screening techniques are costly and ultimately common people have to take the burden of increased payment. Due to the significantly increased cost of the cervical smear screening, the screening program will be unpopular. This may seriously jeopardize the goal of the cervical smear screening program. In most of the developing and underdeveloped countries, there is no good organized cervical screening program and thereby the exact role of automation in these countries is still doubtful and needs critical evaluation.

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CHAPTER 22

Polymerase Chain Reaction

Chapter Contents

INTRODUCTION

Polymerase chain reaction (PCR) is one of the important developments in molecular pathology. With the help of PCR, the specific target portion of the deoxyribonucleic acid (DNA) can be amplified. Polymerase chain reaction is now widely used in both diagnosis and research.

PRINCIPLE

The basic principles of PCR are (**Fig. 22.1, Box 22.1**):

- By applying heat, double-stranded DNA is made to single stranded
- Two oligonucleotide strands or primers are used that are complementary to the 3' ends of each strand of DNA
- The primers attach to the 3' end of the DNA strand
- Taq polymerase helps to extend the DNA strand by incorporating nucleotides.

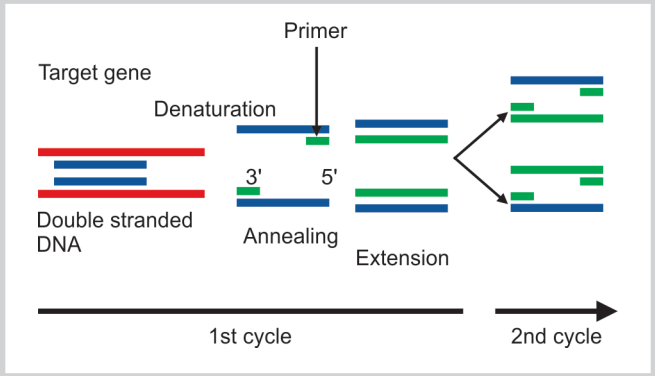

Fig. 22.1: Schematic diagram showing the basic principle of polymerase chain reaction

STEPS OF POLYMERASE CHAIN REACTION

The steps of PCR are (**Box 22.1**):

Denaturation (94°C): DNA is heated to 94°C to make it single stranded. Only 1–2 minutes are given to this heating process in each cycle.

Annealing (54°C): The temperature is rapidly cooled. In this lowered temperature, the primer quickly anneals with the respective site of DNA. With the help of Taq polymerase, the reaction starts at the primer-DNA template site.

Extension (72°C): The complementary nucleotides are attached from the 3' to 5' end of DNA. There is an exponential increment in the number of genes in each cycle. At least 30 cycles of denaturation-annealing-extension is done in each PCR.

BOX 22.1 **Polymerase chain reaction: Principle and steps**

Principle: The target gene is amplified with the help of a pair of primer, Taq polymerase and nucleotides.
Steps:
- Denaturation: Single-stranded DNA is made by heating double-stranded DNA.
- Annealing: Primer binds with the complementary DNA strand in 3′ region.
- Extension: With the help of Taq polymerase and in presence of nucleotides new DNA strand is formed from 3′ to 5′ direction.

At least 30 such cycles occur

BOX 22.2 **Polymerase chain reaction: Applications**

- Diagnosis of infection
  - Viral
  - Bacterial
  - Parasitic
- Cancer
  - To detect base pair mutation
  - To detect chromosomal translocation
  - To detect minimal residual diseases
  - To detect monoclonality of B and T cell in NHL
- Genetic
  - Intrauterine detection of genetic disorder such as Down's syndrome, cystic fibrosis, sickle cell anemia, Gaucher's, thalassemia, hemophilia
  - Parental detection: By detection of tandem repeat in noncoding DNA

TYPES OF POLYMERASE CHAIN REACTION

There are different types of PCR methods for diagnostic purposes. These are:

- Direct polymerase chain reaction: This is the standard PCR method described before.
- Reverse transcriptase polymerase chain reaction: In this method, cDNA is made from ribonucleic acid (RNA) and then cDNA is amplified by PCR technique. Reverse transcriptase polymerase chain reaction is used for RNA targets.[1]
- Asymmetric polymerase chain reaction: In this process, unequal molar concentration of primer is used and single-stranded DNA is made. This is used for DNA sequencing.[2]
- Inverse polymerase chain reaction: Inverse PCR amplifies DNA outside the boundary of the known target sequence. This is particularly employed to find out the clonality of lymphoma and the insertion site of viral DNA.[3]
- Single-strand conformation polymorphism: This method is used to detect single base change mutation in DNA or RNA and uses the property of different mobility of DNA strand having a different base pair.[4]
- In situ polymerase chain reaction: This technique is used to amplify the nucleic acid in the fixed tissue and cell instead in solution. As the cells are intact so the PCR can be correlated with the morphology.[5]
- Real time polymerase chain reaction: Real-time quantitative PCR helps in specific and reproducible quantitation of nucleic acids.[6] In the case of original PCR, the same quantity of the product is generated after the end of the process. In real time PCR, the amount of product can be monitored during each cycle of PCR with the help of a fluorescent dye and therefore it is possible to calculate the number of DNA molecules of the amplified sequence that were initially present in the sample.
- Nested polymerase chain reaction: In case of nested PCR, more than two pairs of primers are used for DNA amplification. The first pair of primer is used as the standard PCR practice and the second pair of primer is used to bind within the first PCR product. This prevents the contamination or wrong amplification of DNA because if any wrong locus is amplified by error then the second time it will not be amplified again by the second PCR primer.

APPLICATIONS OF POLYMERASE CHAIN REACTION

The applications of PCR have been listed in **Box 22.2**.[7,8]

Diagnosis of Infection

- Viral: Polymerase chain reaction is able to diagnose a long list of viruses such as herpes virus, human papilloma virus (**Fig. 22.2**), cytomegalovirus, hepatitis virus, human immunodeficiency virus, Epstein-Barr virus,[9] etc.
- Bacterial: Detection of tuberculosis—conventional diagnosis of the culture of *Mycobacterium tubercle* takes about 6 weeks. However, PCR can diagnose tuberculosis within few hours. It also can diagnose the infection in early and latent phase which is not possible with the conventional laboratory techniques. Other bacterial infections such as *Borrelia burgdorferi*, *Legionella pneumophila*, *Listeria monocytogenes*, *Chlamydia trachomatis* can also be diagnosed by PCR.[9]
- Parasitic infection: Polymerase chain reaction has been used to detect parasitic infections such as *Toxoplasma gondii* and *Plasmodium falciparum*.[9]

Cancer

Polymerase chain reaction has been used widely in cancer genetics. It is used:

- To detect mutation in oncogenes and tumor suppressor genes—p53, c-myc, ras gene[8]
- To detect diagnostic chromosomal translocation in various malignancies. With the help of highly specific primers, various chromosomal translocations can be detected by PCR[8]

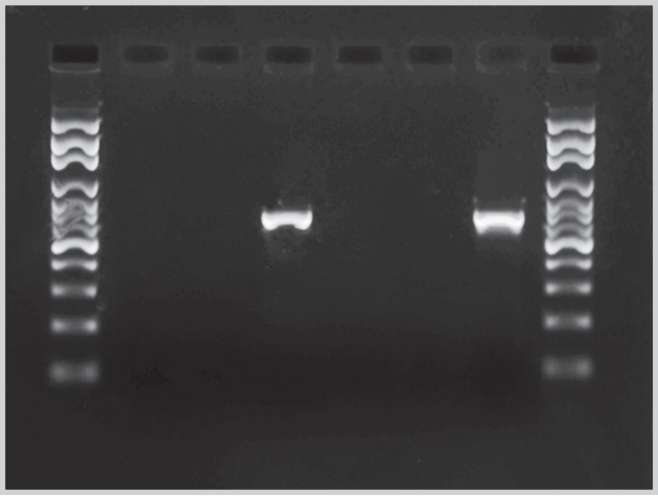

Fig. 22.2: Agarose gel electrophoresis (1.5%) from fine-needle aspiration cytology of lung carcinoma. Lane 1,8-molecular weight marker (100 bp), lane 2-negative control, lane 3, 4, 5, 6-expression of human papilloma virus subtype 16 in Patients, lane 7-positive control (690 bp) *Source:* Dr Ritu Aggarwal, Associate Professor, Department of Immunopathology, PGIMER, Chandigarh

- To detect monoclonality of non-Hodgkin's lymphoma such as T cell receptor gene analysis[10]
- To detect minimal residual disease during follow-up of a patient[11]

Genetic Diseases

With the help of PCR technique, various genetic diseases can be diagnosed such as Down's syndrome, cystic fibrosis, sickle cell anemia, Gaucher's, thalassemia, hemophilia, etc. Polymerase chain reaction technique helps in the diagnosis of many intrauterine genetic diseases of fetus much early without any aggressive placental bed biopsy. Fetal cell can be isolated either from blood or from cervical mucosa of the mother. Polymerase chain reaction of DNA of the fetal cell can be amplified to detect these chromosomal abnormalities.[12]

Polymerase chain reaction may also be helpful to detect tandem repeats in noncoding DNA part of the chromosome and therefore to help in parental identity of the fetus.[13]

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CHAPTER 23

Quality Control and Laboratory Organization

Chapter Contents

INTRODUCTION

Quality control is defined as a set of measures for verifying and maintaining a desired level of quality in an individual test or process,[1] whereas, the quality assurance is defined by College of American Pathologists as "systematic monitoring of quality control results and quality practice parameters to assure that all systems are functioning in a manner appropriate to excellence in healthcare delivery".[2] It is in fact the dynamic process by which the quality control is built up in a laboratory.

Quality control starts from the receiving of the sample to the final delivery of reports and further storage and follow-up of the cases. Quality control can be divided into:

- Preanalytical phase: Before interpretation of slide
- Analytical phase: At the time of interpretation of slide
- Postanalytical phase: After the interpretation of slide.

All these stages are part of internal quality control (IQC) program.

PREANALYTICAL PHASE

Quality control in this stage includes specimen collection, receiving, processing and staining of the sample.[1] At each successive stage, various precautions and measures should be taken such as:

- There should be a laboratory standard operating protocol for specimen collection and processing
- Unique bar code or number should be issued for each specimen. No duplication should be allowed
- All the forms should have the space for detailed clinical complaints, radiological, biochemical data, previous biopsy or cytology report (if any)
- The data in the requisition form should be entered into computer routinely
- The chief of the laboratory should routinely check the processing and staining quality
- The time period between receiving sample and submission of the stained slide should be optimum.

ANALYTICAL PHASE

This is one of the important stages in laboratory service.

Cervical Smears

- All the cervical smears should be screened by qualified cytotechnologists. The primary screener should not screen more than 100 slides in 24 hours time period.[3]
- All the positive slides must be reported by the cytopathologist.[4]
- Negative smear: To decrease the false negative rate in cervical smear screening various rescreening techniques have been recommended.[5,6]

Proportional Rescreening

According to rules of Clinical Laboratory Improvement Amendments of 1988, USA, 10% of all negative cervical smears should be rescreened by the senior cytotechnologists or cytopathologist.[3] This is not a very effective technique[7] and many other techniques have been introduced.

Selected Rescreening

This is the rescreening of selected group of patients who requires special attention. The selected group of patients includes the patients with abnormal bleeding, suspicious cervix on visual examination, HIV-infected patients, etc.

Rapid Review

In this technique, all the cervical cytology smears are rapidly screened by a trained cytoscreener after the initial screening by primary screener. This is currently considered as the most efficient technique in detection of false negative cases.[5]

Automated Rescreening

Automated rescreening is done by the computerized automated system and this has been described in Chapter 21

Nongynecological Smears

In case of nongynecological sample, the laboratory should follow the norms as:[8]

- Every case should be reported by the cytopathologist[9]
- In case of difficulty in particular cases, the reporting cytopathologist should discuss with the fellow colleagues to reach a consensus diagnosis
- During reporting of the cases, the cytopathologists should follow the consistent reporting format
- One should be careful about using terminology such as "suspicious", "highly suspicious", "suggestive" and "consistent with", etc.

POSTANALYTICAL PHASE

It is very important to have further follow-up result of the reported cases as far as possible. For IQC of the laboratory the following measures should be taken.

Cytology-Histology Correlation

- Cytohistology correlation: Cytological diagnosis should always be correlated either with histology or clinical follow-up or autopsy
- All the discordant cases should be reviewed carefully and both cytology and histology slides should be seen
- During review of the discrepant cases, one should avoid bias. This can be done by:
  - A third person should screen the slide without any knowledge of clinical outcome
  - The discrepant cases could be introduced among the routine slides.

Computerized Record

There should be complete computerized record of all the cytology and histology cases. The data and slides should be kept for at least 10 years.[10]

Statistical Analysis and Performance Measurement

Periodic statistical analysis of the cytohisto correlation is mandatory for quality control. There are various ways to measure the performance of the test.

Sensitivity: It means the percentage of actual (true) positive cases reported among all the positive cases.

$$\text{Sensitivity} = \frac{\text{True positive}}{\text{True positive + false negative}} \text{ X } 100\%$$

Specificity: It means the percentage of truly negative cases reported among all the negative cases.

$$\text{Sensitivity} = \frac{\text{True negative}}{\text{True negative + false positive}} \text{ X } 100\%$$

False positive: It is defined as a diagnostic test that is reported as malignant, however, the patient shows a benign tumor or no tumor at all. The false positive report is described differently by different persons and there is a lack of uniformity in definition.

False negative: False negative diagnosis means that in a diseased individual the diagnosis is either completely missed in the report or the severity of the disease process is underestimated.

False negative proportion: It is defined as the fraction of the patients missed among the total number of abnormal cases.

$$\text{False negative proportion} = \frac{\text{False negative report}}{\text{True positive + False negative}}$$

Record keeping: This is also a part of the IQC. All the slides should be kept at least for 10 years for future review.[10] Slides should be arranged orderly so that any slide could be retrieved as quickly as possible. In some laboratories, the negative report is kept only for 3 years for shortage of space.

External Quality Assurance

Proficiency test and continuing medical education: The proficiency test is not mandatory for the laboratory personnel who examine gynecological samples. However, it is preferable that all the

cytotechnologists should appear in proficiency evaluation test. There should be option for retraining and retesting the laboratory staff.

Continuing Medical Education

There should be adequate facilities or options for laboratory staff to take part in continuing medical education, workshop and symposium so that they are able to improve their knowledge and efficiency.

LABORATORY ORGANIZATION

Laboratory organization of a cytology laboratory, has the following components:

- Laboratory personnel
- Building, instrument and safety precautions
- Organization infrastructure and system protocol

Laboratory Personnel

In each cytology laboratory, the following staff is needed to maintain the smooth quality work:

- Consultant cytopathologist
- Senior cytotechnologist or biomedical scientist
- Cytology screeners
- Technical laboratory personnel
- Secretarial and clerical staff.

Consultant Cytopathologist

The consultant cytopathologist should be qualified pathologist with adequate experience in cytology. The duties of the cytopathologist are:

- Final reporting: He/she is the final reporting authority and should assess and sign all the cases
- Screening: The consultant cytopathologist should examine all the abnormal cases and certain proportion of negative cases to maintain the accuracy and quality of the work in the laboratory
- Cytohisto correlation: He/she should try to correlate cytology-histology and review all the discrepant cases. He/she should maintain a regular audit of the laboratory cases.
- Communication with clinicians: He/she should maintain communication between the clinician and the laboratory staff. He/she should take part in clinicopathological meeting, and inform the clinician about the discrepant cases, give an opinion on individual cases regarding further investigations or management.
- Teaching and training: The consultant cytopathologist should educate the laboratory technologists by regular teaching and training and arrange continuing medical education program. Cytopathologist should also teach and train the postgraduate medical students in cytology. He/she should also encourage the nonmedical clerical staff for further training.

Senior Cytotechnologist or Biomedical Scientist

The senior cytotechnologist should have adequate experience in gynecological cytology. The duties and responsibilities of senior cytotechnologist are:

- Management and supervision of all the junior cytotechnologists
- Screening certain proportion of negative cases and should review all abnormal cases
- He/she should act as a communication link between the other junior cytotechnologists and consultant cytopathologist
- Senior cytotechnologist may have to manage daily laboratory services and also personal affairs of all the staffs.

Cytology Screeners

The cytology screener should have:

- Adequate training on screening cytology slides
- Cytology screener can screen both gynecological and nongynecological smears. However, all the cases of nongynecological smears should be re-examined by the cytopathologist for final reporting
- In addition, the cytology screeners should also do routine cytology laboratory work, if needed.

Technical Laboratory Personnel

There should be an adequate number of trained technical personnel in the laboratory. The laboratory technical staff should be able to do the routine laboratory work. They should take part in a quality control program.

Secretarial and Clerical Staff

The secretarial and clerical staff should be efficient enough to receive the sample, enter the data and dispatch the report. All the staff should be computer literate and should be acquainted in medical terminologies.

Laboratory Building and Instrument

- Location: Proper location of the laboratory is very important. So the laboratory should be located and built in such a way that its function can be performed properly.
- Rooms: There should be separate rooms for reception and secretarial staffs, processing and staining and screening or reporting.

Specimen Processing and Staining Area

This area should be spacious, well-lighted and well-ventilated. The sink for handwash should be separated from the sink used for laboratory samples. There should be proper place or racks to store chemicals, particularly toxic chemicals should be in separate places.

Specimen Preparation Area

This room should have a Class I microbiological safety cabinet for processing of nongynecological samples. In addition, this room must contain centrifuge machine, cytocentrifuge, refrigerator and autoclave machine.

The benches for slide preparation should be in proper height to prevent excessive bending, etc.

Screening Room

The screening room or reporting room should be properly ventilated, spacious and pleasant. There should be comfortable chairs and tables for screening or reporting slides. This room should be free from any noise. The cytoscreener should have proper privacy in this room.

Secretarial and Office Room

This room should have adequate light and enough space for computer, printer and other necessary equipments. The staff should have comfortable furniture and comfortable area for work.

Organization Infrastructure and System Protocol

Infrastructural organization of a laboratory is important for smooth workflow and overall quality work. **Figure 23.1** shows the workflow of a laboratory. All specimens should be received by the clerical staff and should be transferred to the laboratory staff after entering the data and giving a specific unique bar code. The sample should be processed and stained according to the standard laboratory protocol by the medical laboratory assistant. The stained smears should be screened by the cytoscreener. All the abnormal smears and a certain proportion of negative smears should be checked by the senior cytotechnologist. In case of rapid review of negative cases, the senior cytotechnologist should screen all the cases rapidly. Finally, the cytopathologist should examine and report all the abnormal smears. In case of nongynecological cases, the consultant cytopathologist should report all the cases. Finally, the computerized typed report should be signed manually or electronically by the consultant cytopathologist and delivered to the patient.

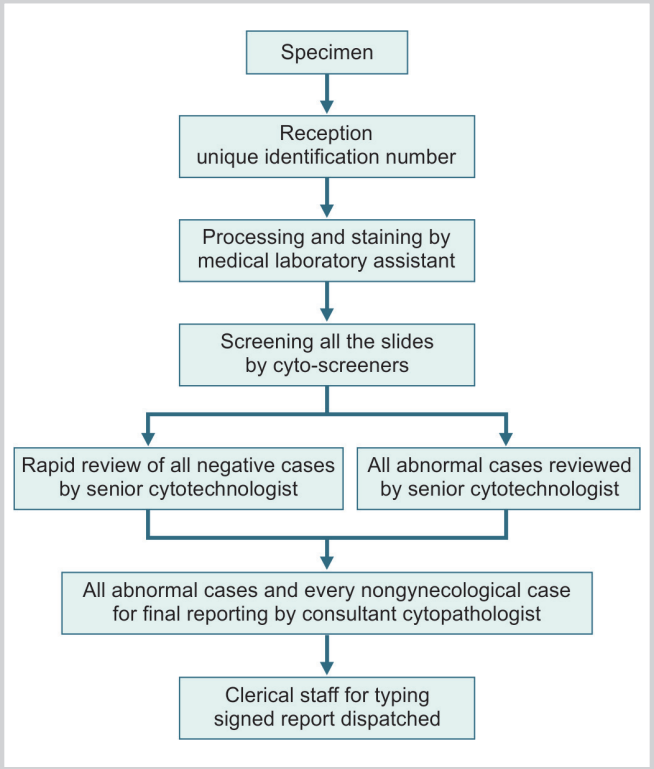

Fig. 23.1: Schematic diagram showing workflow of the cytology laboratory

LABORATORY SAFETY

Laboratory safety should be the prime importance for the entire staff working in the laboratory. Following matters should be kept in mind for the proper safety of the laboratory:

- Security
- Fire hazards
- Chemical hazards
- Infective
- Waste disposal

Security

- Proper security of the laboratory staff, chemicals and valuable equipments are mandatory
- Entry of unauthorized person should be restricted to the laboratory.

Fire Hazards

- All the laboratories should have fire extinguishers and smoke alarms
- The staff should know how to use the fire extinguisher
- Explosive or inflammable chemicals should be handled with adequate precautions.

Chemical Hazards

- All the chemicals should be kept in proper place particularly inflammable chemicals and they should be kept in a fireproof metal cabinet
- All the chemicals should be kept in respective original bottles
- Suction by mouth to draw material should not be allowed
- Laboratory staffs should wear proper gloves, laboratory coat, etc. during handling of chemicals
- During preparing diluted solution, the concentrated acid or alkali should be added in water.

Infective

In this era of HIV infection, every laboratory sample should be handled cautiously and unless specified the sample should be

considered as a potential source of infection. The laboratory staff should follow universal precautionary measures.[11]

- Barrier precautions
  - Gown: All healthcare personnel should wear proper laboratory gowns. The gown should cover the full arms and the front of the body from neck to mid thigh.
  - Mask: Mask may be used in combination with goggles to protect the face and eyes and also to prevent droplets or aerosol infection.
  - Gloves: Gloves are used to prevent infection, contamination by hands. Hygienic measures of the hand after removal of the gloves further help to prevent contamination particularly if the person has a cut in hand.
  - Goggles: To prevent eye infection.
- Precautions from the sharp objects: Following precautions should be taken to prevent injury by sharp objects such as needles, scalpel, etc.
  - The needles should not be recapped or manipulated by hand
  - Sharp objects should be placed in appropriate containers
  - Needle cutter should be used to destroy the needle.
- Hand hygiene: If hands are contaminated with blood or other body products then they should be washed immediately with the antiseptic soap and water. Alcohol-based products such as gel or foam is better to wash as they do not require any water.
- Prevention from mucus membrane contact: Exposure to the mucus membrane of eyes, mouth or nose should be avoided. In addition to wearing protective barrier such as mask, gloves, etc. one should always avoid mouth to mouth resuscitation during emergencies and other mechanical ventilation devices should be used.
- Duty of the sick person: Healthcare workers with weeping dermatitis or wound in the hand should not work for direct healthcare.
- Common norms in the laboratory: In addition, the laboratory workers should follow certain norms to prevent infection:
  - Eating, drinking or smoking should not be allowed in the laboratory.
  - Contaminated material, syringes, fluids, etc. should always be placed in proper containers
  - Specimen preparation of high-risk substances should always be done in biological safety cabinet
  - Laboratory personnel should always wash their hands and face after laboratory duty.

Waste Disposal

Laboratory waste material may be of two broad types: (1) general waste material and (2) biohazardous waste.

- Label: All waste material should be kept in properly labeled waste disposal container in a proper colored container as mentioned below.
- Storage of waste: Closable, puncture-resistant, leak-proof container should be used.
- Accumulation of laboratory waste: Only one container is used for each kind of waste material. The waste material should be disposed before it completely fills the container.
- Container: The container for sharper objects should be closable, puncture-resistant and leak-proof. The colors of the containers and their contents (**Fig. 23.2**) have been described below:
  - Biological contaminated wastes: This includes microbiological wastes and contaminated body fluids such as urine, sputum, blood, etc.
    - Color of container: Yellow
    - Type of container: Plastic bag
    - Treatment: This should be treated with disinfectant followed by autoclaving.
  - Sharp objects: This includes needles, scalpel, broken glass, etc.
    - Color of container: Blue
    - Type of container: It should be closable, puncture-resistant and leak-proof hard plastic container.
    - Treatment: Incineration
  - Solid contaminated with human waste materials: This includes syringes, catheters, etc.
    - Color of container: Red
    - Type of container: Plastic bag
    - Treatment: Disinfected followed by incineration
  - Low level chemically contaminated waste: Unused medicines, papers, various chemicals used for disinfection:
    - Color of container: Black
    - Type of container: Plastic bag
    - Treatment: Local authority for routine waste disposal.

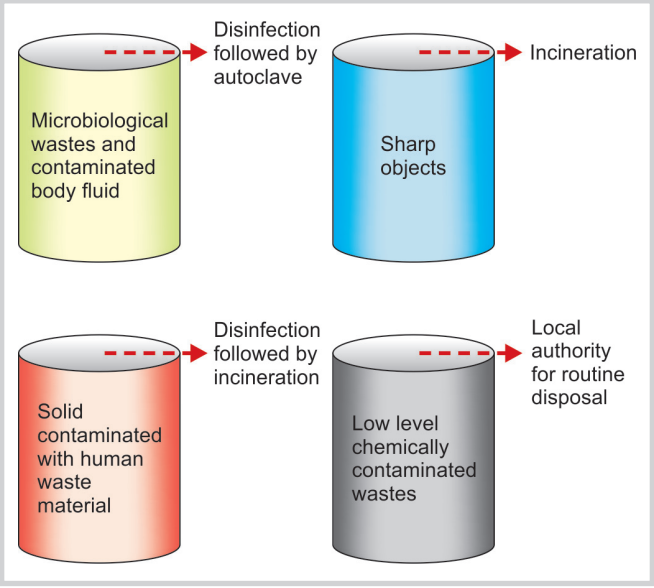

Fig. 23.2: Schematic diagram showing color of the bags and methods of waste disposal

A DISINFECTANT USED IN FOR THE CONTAMINANTS

Sodium Hypochlorite

This is a fast acting oxidant and a broad spectrum chemical disinfectant. It is diluted with water to get optimal strength. An aqueous solution 1:10 dilution of sodium hypochlorite may serve the purpose of disinfection in the laboratory. The solution should be prepared daily. Chlorine is liberated from the solution that is highly corrosive. Therefore, the solution should not be kept in metal containers.

Many other chemical germicides such as quaternary ammonium compounds (cetrimide, Savlon, etc.), iodine and iodophors, hydrogen peroxide, etc. can also be used for laboratory disinfection.

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SECTION 4

Fine Needle Aspiration Cytology

CHAPTER 24

Head, Neck and Orbit

Chapter Contents

HEAD AND NECK

Large varieties of primary and metastatic tumors from the different tissues may occur in the head and neck region. Fine needle aspiration cytology (FNAC) is widely used to detect the primary and recurrent tumors in this region. It can be taken from the multiple sites and also from the distorted scar sites after radiation of the primary malignancies. It is also important to have thorough knowledge of the non-neoplastic lesions in this region that may mimic as malignant tumors.

CYSTIC LESIONS

The cysts may be congenital or acquired (**Box 24.1**). Various neoplastic lesions may also present as cyst in the head and neck region. It is important to note that metastatic squamous cell carcinoma (SCC), papillary carcinoma of thyroid in the lymph node and a large number of parotid tumors are often cystic. There are chances to have false positive or negative diagnosis and adequate cares must be taken to avoid the mistake.

Branchial Cyst[1]

The branchial cyst is one of the common non-neoplastic cysts in the lateral neck region. They are usually noted along the medial border of the sternocleidomastoid muscle. This cyst possibly develops from the congenital remnants of the branchial cleft.

Cytology (Fig. 24.1)

Aspiration of the cyst usually yields turbid fluid. The smears from the centrifuged fluid specimen show many discrete squamous cells, occasional clusters of columnar cells and foamy histiocytes (**Box 24.2**). Squamous cells often show reactive changes such as nuclear enlargement and mild degree of pleomorphism.

BOX 24.1 **Cysts in head and neck region**

Congenital:
- Branchial cyst
- Thyroglossal cyst
- Cystic hygroma

Acquired:

Non-neoplastic:
- Dermoid cyst
- Cysts of the jaw
- Salivary gland retention cyst
- Mucocele

Neoplastic:
- Metastatic squamous cell carcinoma
- Warthin's tumor
- Acinic cell carcinoma
- Pleomorphic adenoma

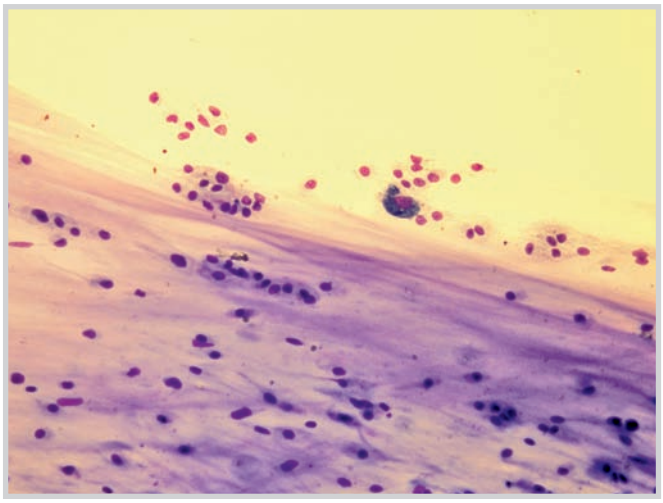

Fig. 24.1: Branchial cyst: Columnar cells and foamy histiocytes in the background of mucinous material [May-Grünwald-Giemsa (MGG) stain × medium power (MP)]

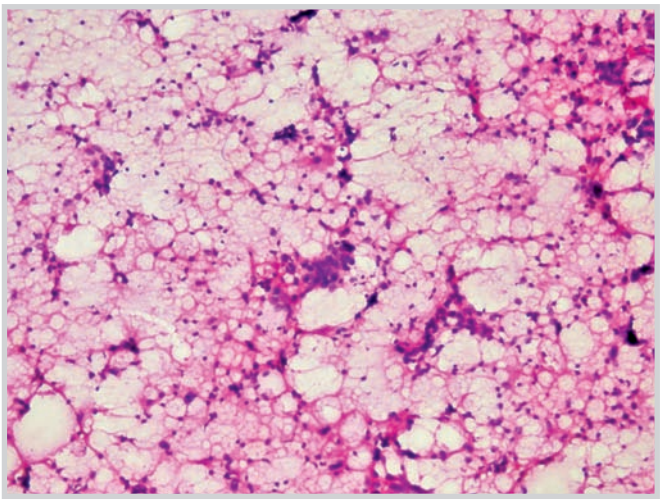

Fig. 24.2A: Cytology smear of cystic squamous cell carcinoma showing multiple foamy histiocytes and scattered malignant squamous cells [hematoxylin and eosin (H & E) stain × MP]

BOX 24.2 Branchial cyst

- Turbid fluid
- Benign squamous cells
- Columnar cells
- Foamy macrophages
- Cholesterol crystals

Background of the smear shows mucinous material and cholesterol crystals.

Metastatic SCC in the lymph node often shows cystic degeneration. FNAC of such lesions yields brownish fluid and smears of which show mature-looking squamous cells and polymorphs in a thin necrotic background (**Figs 24.2A and B**). Cytology smears of such cases are the potential sources of misdiagnosis. The following features may be helpful in diagnosis of branchial cyst:

- Young patient
- Long history
- Bland looking nuclear chromatin
- Minimal nuclear pleomorphism

Differential Diagnosis

Metastatic SCC.[2]

Thyroglossal Cysts

Thyroglossal cysts are commonly present as midline neck cysts. During embryogenesis, the thyroid comes down from the foramen of cecum of the tongue and thyroglossal duct is formed. Later on, this duct obliterates. Dilatation of the persistent thyroglossal duct causes the formation of the thyroglossal cyst.

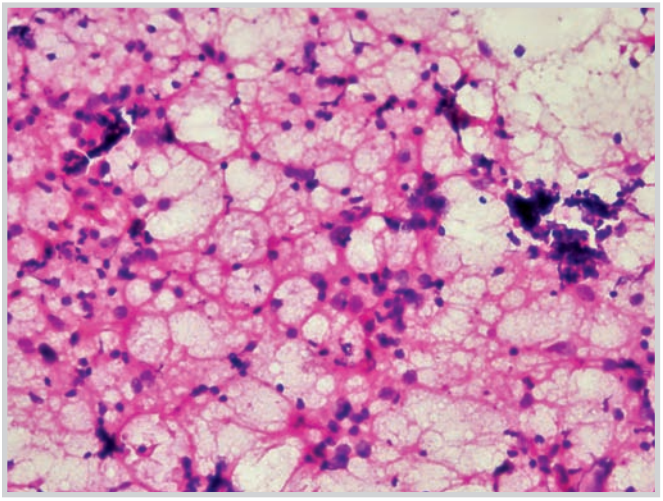

Fig. 24.2B: High powered view of the cytology smear of cystic squamous cell carcinoma showing histiocytes with foamy cytoplasm and squamous cells with hyperchromatic enlarged nuclei [H & E × high power (HP)]

Cytology

Fine needle aspiration cytology of the thyroglossal cyst yields colloid (**Box 24.3**). The smear shows many ciliated columnar cells and occasional squamous cells. In addition, thyroid follicular cells may also be seen in the background. Without the precise location of the tumor, it may be difficult to differentiate thyroglossal cyst from the colloid goiter.

Epidermal Inclusion Cyst

Epidermal inclusion cyst may occur anywhere in the head and neck region. FNAC yields thick whitish material. The smears

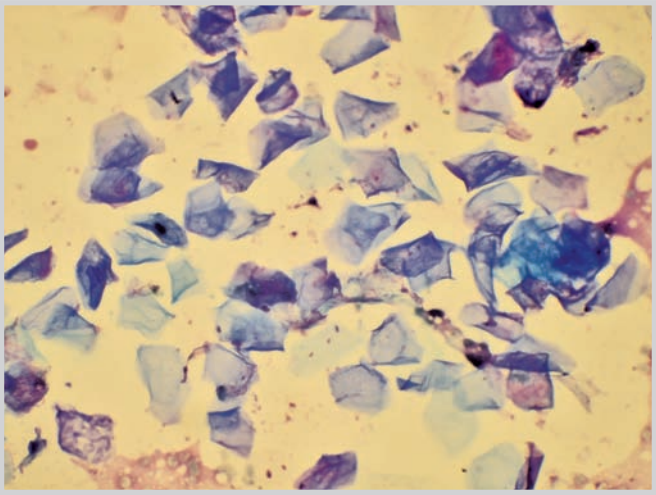

Fig. 24.3: Fine needle aspiration cytology (FNAC) smear of epidermal inclusion cyst showing abundant anucleated squamous cells (Papanicolaou's stain × HP)

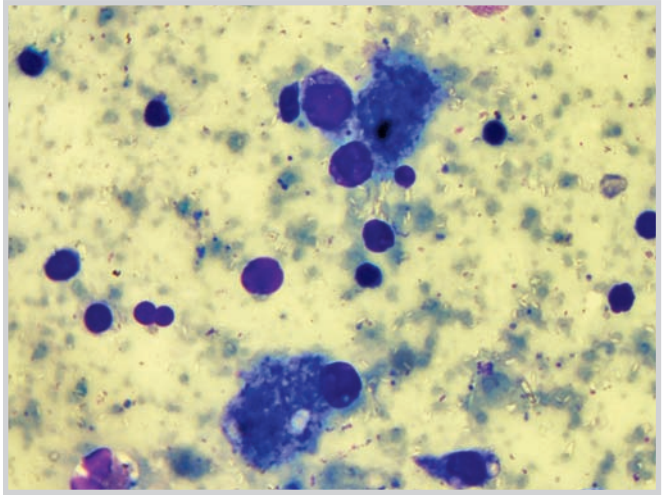

Fig. 24.4A: Cystic hygroma: Lymphocytes and foamy macrophages (MGG × HP)

BOX 24.3 Thyroglossal cyst

- Ciliated columnar cells
- Squamous cells
- Occasional thyroid follicular cells
- Background colloid

show many discrete benign squamous cells and anucleated squamous cells (**Fig. 24.3**). At times these cells may show nuclear enlargement and pleomorphism. This type of reactive changes should always be distinguished from the metastatic SCC. Occasional multinucleated giant cells are also seen.

Cystic Hygroma

Cystic hygroma is large diffuse lymphangioma that predominantly occurs in the infant and young adults. FNAC usually yields thin fluid. The smears show many discrete lymphocytes (**Fig. 24.4A**).

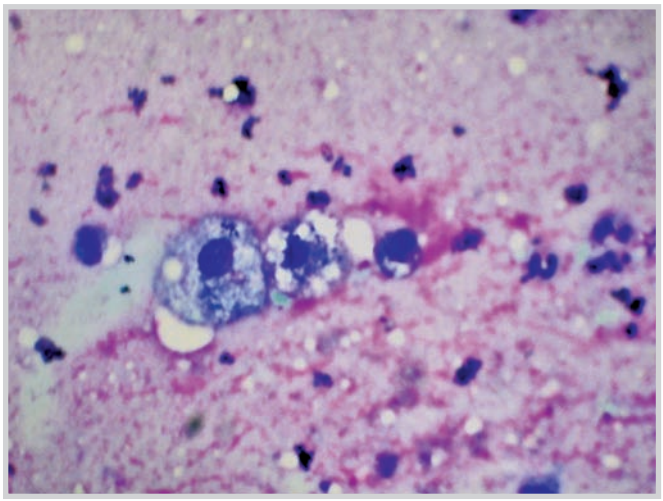

Fig. 24.4B: Fine needle aspiration cytology (FNAC) smear of mucocele showing abundant mucoid material and foamy vacuolated histiocytes (MGG × HP)

Mucocele

This is due to the extravasation of fluid from the retention cyst of the minor salivary gland to the neighboring soft tissue. FNAC yields thick mucinous fluid and the smears of which show many histiocytes (**Fig. 24.4B**).

NEOPLASTIC LESIONS

Paraganglioma[3]

Carotid body paraganglioma is a neuroendocrine tumor that originates from the glomus jugulare of the neck. It is also known as carotid body tumor. The carotid body tumor locates in the bifurcation of the common carotid artery. The patient presents as a slow growing mass under the sternocleidomastoid muscle. The swelling is pulsatile and is movable side to side but not in vertical direction. It is often recommended that FNAC should not be done from this tumor as there is a chance of local hemorrhage and cerebral catastrophy after FNAC. However, we have not seen any such complications so far.

Cytology (Figs 24.5 to 24.9)

Fine needle aspiration cytology yields predominantly blood mixed material. The smear shows small loosely cohesive clusters and dissociated tumor cells (**Box 24.4**).

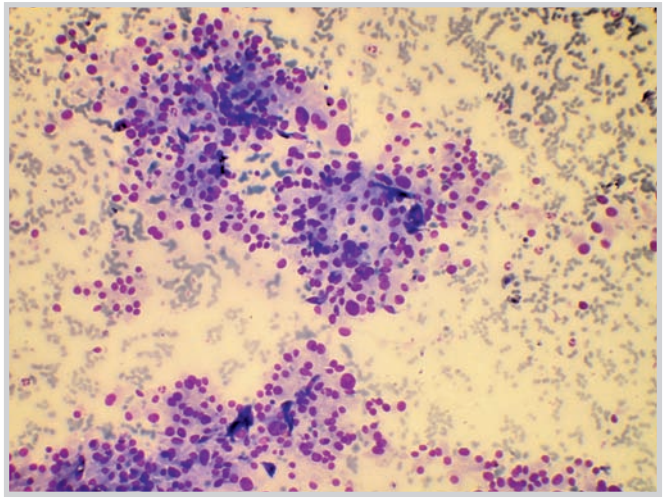

Fig. 24.5: Loose clusters and dissociated cells in fine needle aspiration cytology (FNAC) smear of paraganglioma (MGG × MP)

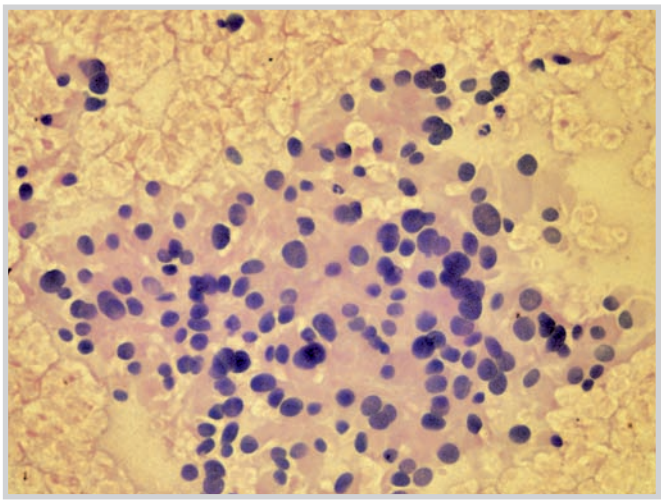

Fig. 24.6: Round to oval cells with indistinct cytoplasmic margin in paraganglioma (H & E × MP)

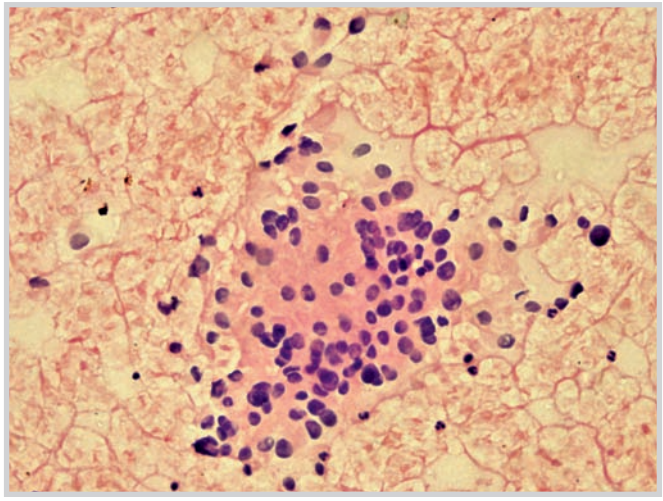

Fig. 24.7: Round to oval cells with moderate nuclear pleomorphism in paraganglioma (H & E × MP)

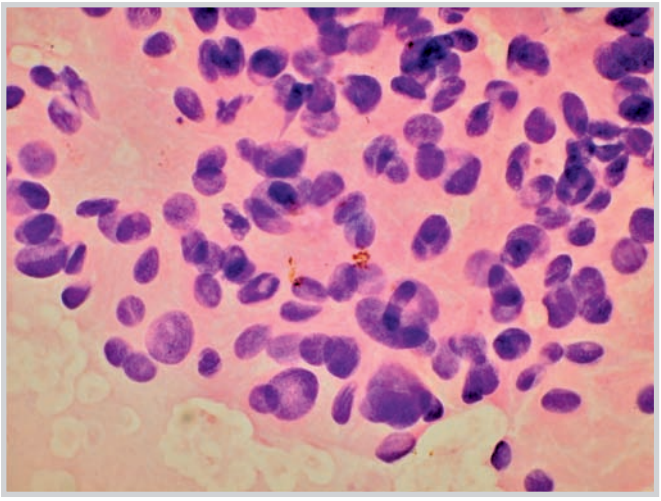

Fig. 24.8: Ill-formed rosette-like structures in paraganglioma (H & E × HP)

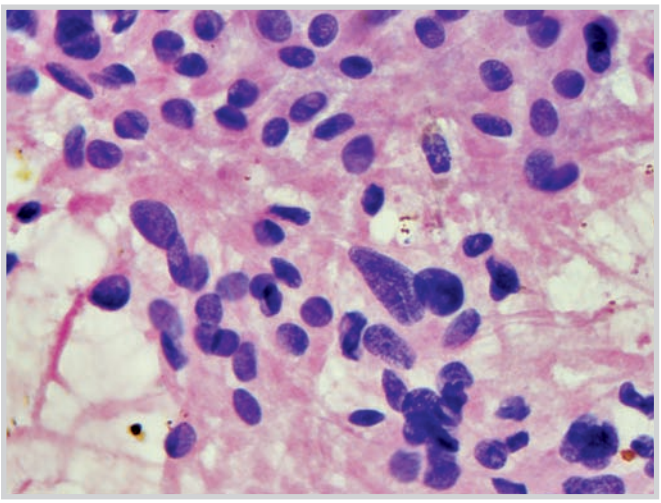

Fig. 24.9: Cells with bland chromatin in paraganglioma (H & E × HP)

Occasional rosette-like arrangement may be seen. The individual cells show moderate amount of cytoplasm with ill-defined cell border. The nuclei are round to spindle-shaped and are relatively monomorphic in shape. Salt and pepper-like nuclear chromatin is seen. Occasional cells show nuclear pleomorphism among the monomorphic nuclei. The presence of intranuclear inclusions has also been described in paraganglioma.

Immunocytochemistry: The tumor cells are positive for synaptophysin, neuron-specific enolase (NSE) and chromogranin; and are negative for cytokeratin, carcinoembryonic antigen, S-100 protein and calcitonin.

Differential Diagnosis

- *Medullary carcinoma*: Both medullary carcinoma and paraganglioma have overlapping cytological features. However, medullary carcinomas are positive for calcitonin.

BOX 24.4 Paraganglioma

- Syncytial clusters and dissociated cells
- Round to spindle cells
- Cells with indistinct cytoplasmic margin
- Monomorphic nucleus
- Occasional cell shows nuclear atypia
- Bland nuclear chromatin
- Intranuclear inclusion
- Rosette-like structures

- *Carcinoid*: Metastatic carcinoid tumors may simulate as paraganglioma.
- *Metastatic carcinoma*: Marked nuclear pleomorphism in occasional cells of paraganglioma may simulate metastatic carcinoma. However, the nuclear chromatin is bland in paraganglioma.

Nasopharyngeal Carcinoma[4]

Nasopharyngeal carcinoma (NPC) develops from the nasopharyngeal mucosa. It is also known as lymphoepithelioma. It includes nonkeratinizing SCC and basaloid SCC. There is a strong constant association of NPC and Epstein-Barr virus (EBV) infection. This indicates the oncogenic role of this virus. NPC commonly presents with metastatic lymph nodal mass. The patients may also have postnasal blood stained drip, serous otitis media and headache. The tumor has a bimodal age incidence.

Cytology (Figs 24.10 to 24.12)

Fine needle aspiration cytology is usually done from the lymph nodal neck mass of the nasopharyngeal carcinoma. The smears show abundant reactive lymphoid cells admixed with syncytial or dissociated malignant cells (**Box 24.5**). The tumor cells contain moderate amount of cytoplasm with ill-defined border. Nuclei of the cells are round with mild pleomorphism, fine chromatin and single to multiple prominent nucleoli. Prominent macronucleoli are also noted. At times binucleated cells with macronucleoli may simulate Reed-Sternberg cell.

Differential Diagnosis

- *Non-Hodgkin's lymphoma (NHL)*: Abundant lymphocytes along with the dispersed round malignant cells may simulate NHL.
- *Hodgkin's lymphoma (HL)*: Large binucleated cells with prominent nucleoli may resemble Reed-Sternberg cells of Hodgkin's lymphoma.
- *Metastatic small cell carcinoma*: Small cell carcinoma shows cells with scanty cytoplasm, hyperchromatic nuclei, nuclear molding and crushing artifact.

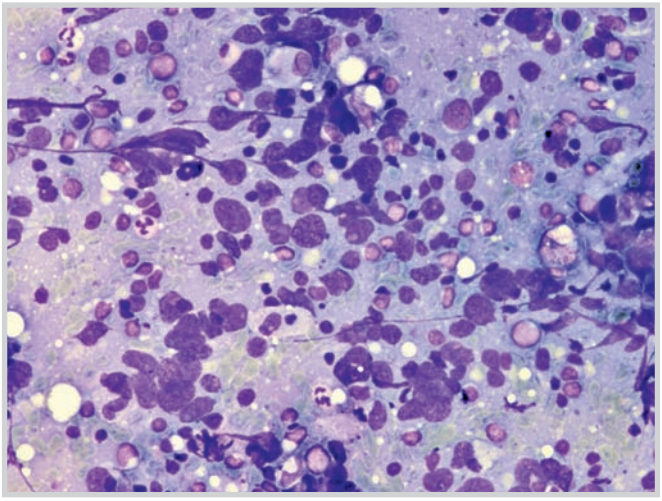

Fig. 24.10: The smears show reactive lymphoid cells admixed with dissociated malignant cells in nasopharyngeal carcinoma (MGG × MP)

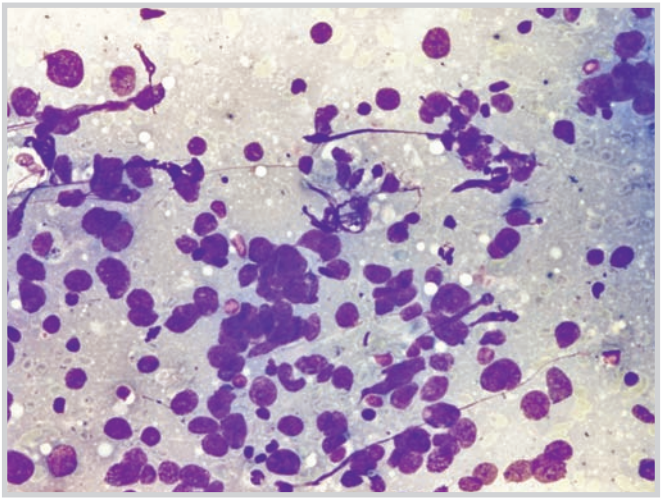

Fig. 24.11: Malignant cells with enlarged moderately pleomorphic nuclei having prominent nucleoli in nasopharyngeal carcinoma (MGG × HP)

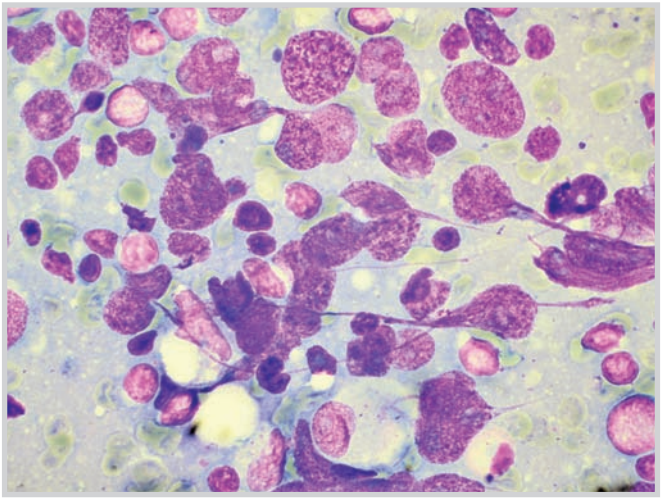

Fig. 24.12: Large cells with fine chromatin and prominent nucleoli in nasopharyngeal carcinoma (MGG × HP)

BOX 24.5 **Nasopharyngeal carcinoma**

- Syncytial or dissociated cells
- Abundant lymphoid cells in the background
- Round to oval cells
- Indistinct cytoplasmic border
- Mild nuclear pleomorphism, fine nuclear chromatin and single to multiple prominent nucleoli
- Immunochemistry: Positive for pan-cytokeratin (AE1/AE3), high molecular weight cytokeratin (CK5, CK6)

Ameloblastoma[5]

Ameloblastoma develops from the odontogenic epithelium of the maxilla. It is a locally aggressive neoplasm and usually occurs in the third to fifth decades of life. The overall incidence of ameloblastoma is less than 1% of all cystic lesions of jaw.

Cytology (Figs 24.13 and 24.14)

Fine needle aspiration cytology smears show predominantly two types of cells: (1) Basaloid cells and (2) squamous cells. The basaloid cells are usually arranged in tight clusters and cords (**Box 24.6**). The cells are arranged in peripheral palisading manner. These cells have scanty cytoplasm and round to oval monomorphic nuclei. Discrete squamous cells are also seen along with occasional squamous pearls. The squamous cells show abundant cytoplasm and centrally placed nuclei. The background of the smear shows loose spindle cells and eosinophilic granular material.

Parathyroid Tumors[6,7]

Parathyroid glands are located behind the thyroid gland. They are total four in number. Parathyroid tumors are nonpalpable and ultrasound or computed tomography (CT) scan is needed for their demonstrations. Parathyroid adenomas may develop in any age but commonly occurs in the fourth decade of life. The cases of parathyroid adenomas are usually present with the features of hyperparathyroidism. The clinical presentations of hyperparathyroidism are the presence of brown tumor, renal problems, and increased serum calcium and parathormone level.

Cytology (Figs 24.15 to 24.18)

Fine needle aspiration cytology smears of parathyroid neoplasm show loosely cohesive clusters, papillary structures and microfollicular pattern (**Box 24.7**). The tumor cells are round with moderate amount of pale vacuolated cytoplasm. The cell shows mildly pleomorphic nuclei and inconspicuous nucleoli. The nuclear chromatin is finely granular. Occasional cells show marked nuclear pleomorphism. In fact, the absence of colloid and cells with abundant eosinophilic or clear cytoplasm should raise the suspicion of parathyroid adenoma.

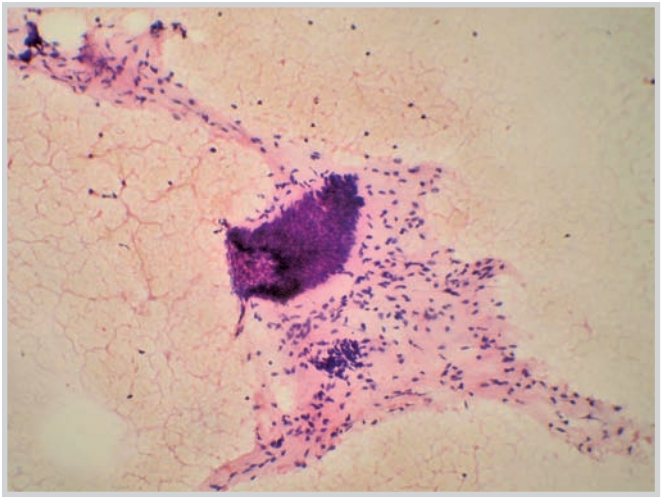

Fig. 24.13: Tight cohesive cluster of basal cells surrounded by loose connective tissue stroma in ameloblastoma (H & E × MP)

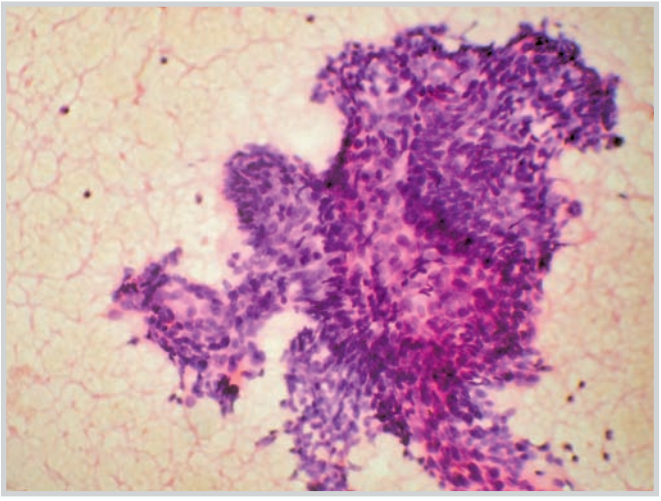

Fig. 24.14: Basaloid cells are arranged in peripheral palisading manner in ameloblastoma (H & E × MP)

BOX 24.6 **Ameloblastoma**

- Basaloid cells
  - Tight clusters and cords
  - Palisading arrangement
  - Scanty cytoplasm
  - Round to oval monomorphic nuclei
- Squamous cells
- Squamous pearls

Predominant population of dissociated cells with marked nuclear pleomorphism, frequent mitosis and the presence of necrosis should raise the suspicion of malignant parathyroid tumor.

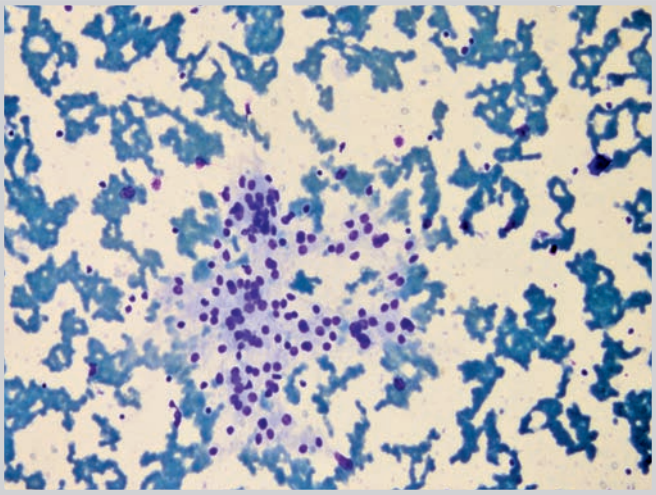

Fig. 24.15: Fine needle aspiration cytology (FNAC) smear of parathyroid neoplasm shows loosely cohesive clusters of cells [MGG × low power (LP)]

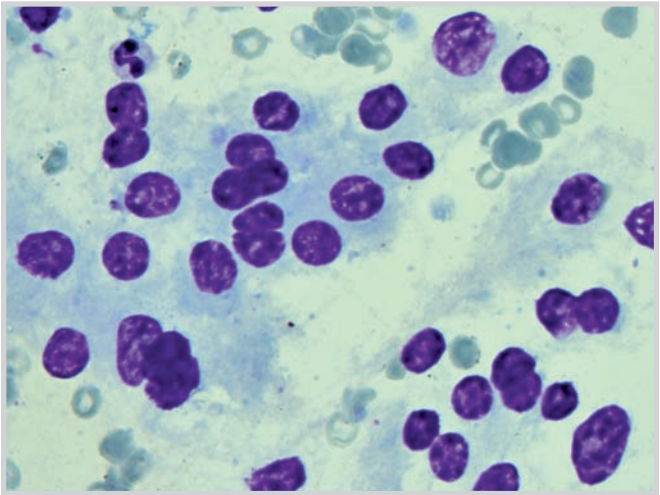

Fig. 24.17: Vague follicle-like arrangement in parathyroid neoplasm (MGG × HP)

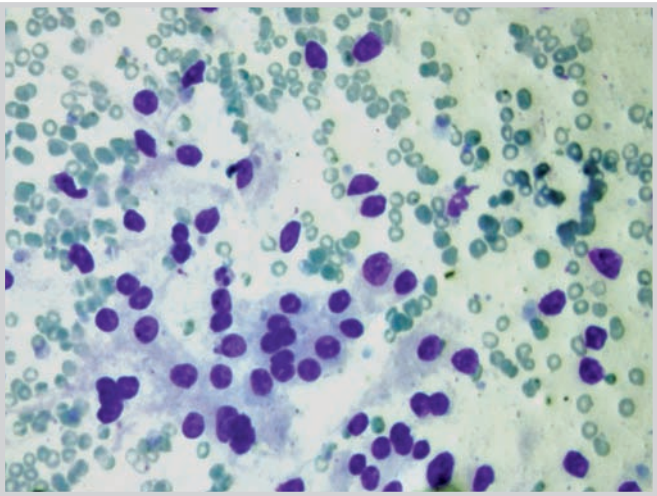

Fig. 24.16: The cell shows mildly pleomorphic nuclei and inconspicuous nucleoli in parathyroid neoplasm (MGG × MP)

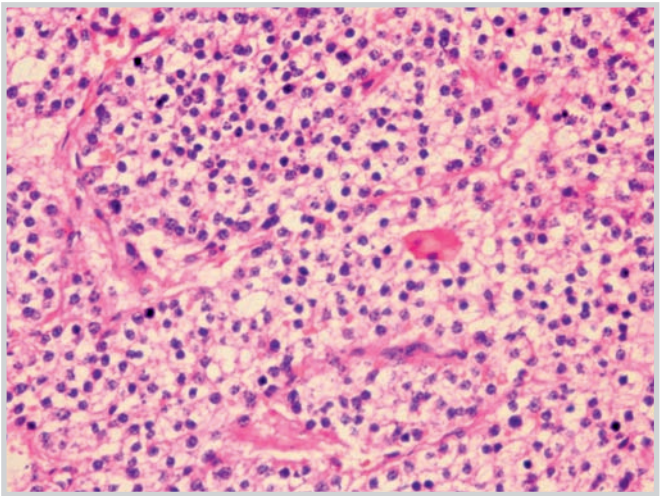

Fig. 24.18: Histopathology section of a parathyroid adenoma showing cells with clear cytoplasm and monomorphic nuclei (H & E × MP)

Differential Diagnosis

Thyroid tumors: Without proper clinical history it is often difficult to suspect parathyroid neoplasm.

Meningioma[8]

Meningioma is rarely noted in the nasal cavity as a protruding mass in the superior part of the nasal cavity and also in the posteriosuperior orbital region.

Cytology (Figs 24.19 to 24.23)

Fine needle aspiration cytology smears show predominantly tight clusters and dissociated cells. The tumor cells show syncytial pattern and whorling appearance (**Box 24.8**). The cells at the periphery of the whorls are flattened, with spindly, condensed nuclei. The tumor cells have moderate amount of pale eosinophilic cytoplasm and mildly pleomorphic nuclei. The nuclear chromatin is bland. Occasional nuclear grooving, intranuclear inclusions and psammoma bodies are noted.

BOX 24.7 **Parathyroid neoplasm**

- Loose aggregates and microfollicular pattern
- Small cells
- Vacuolated clear cytoplasm
- Monomorphic nuclei with salt and pepper chromatin
- Marked nuclear pleomorphism in occasional cells

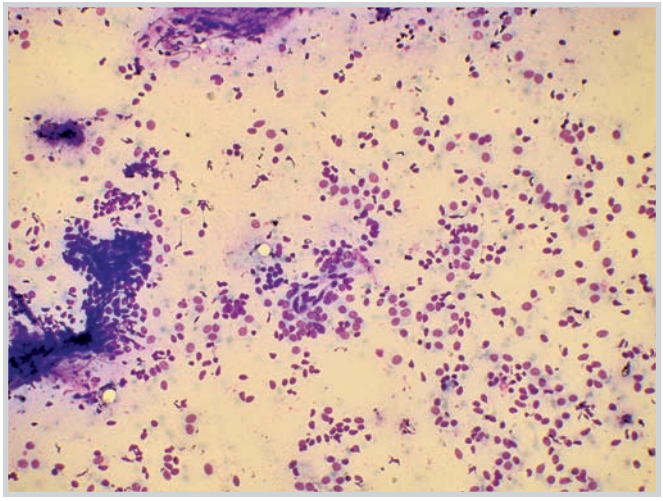

Fig. 24.19: Fine needle aspiration cytology (FNAC) smear showing tight clusters and dissociated cells in meningioma (MGG × MP)

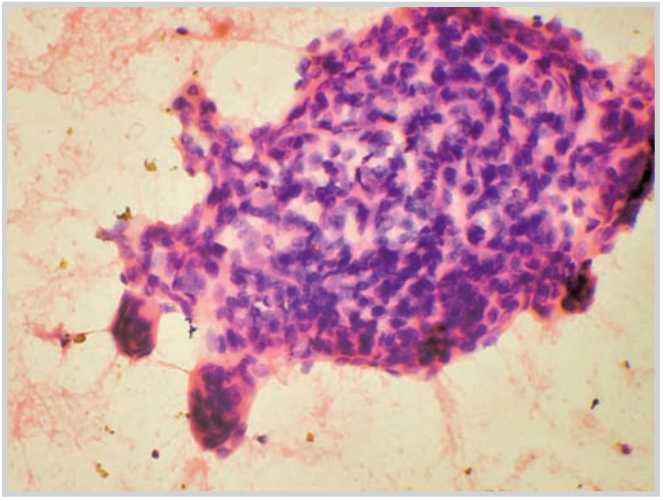

Fig. 24.20: Whorling arrangement of cells and multinucleated cells in meningioma (H & E × MP)

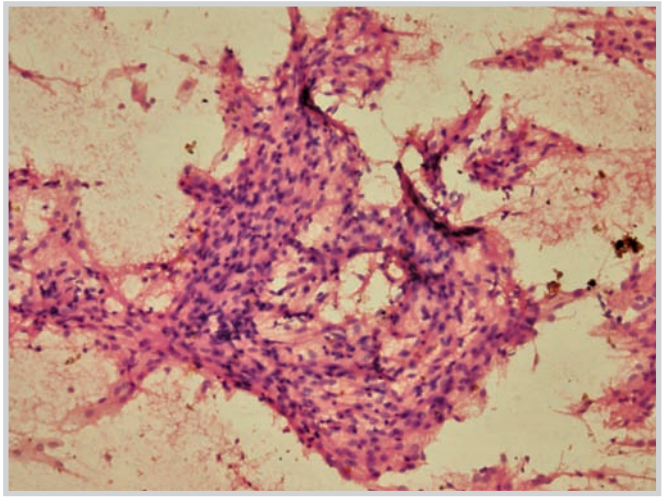

Fig. 24.21: Syncytial arrangement of cells in meningioma (H & E × MP)

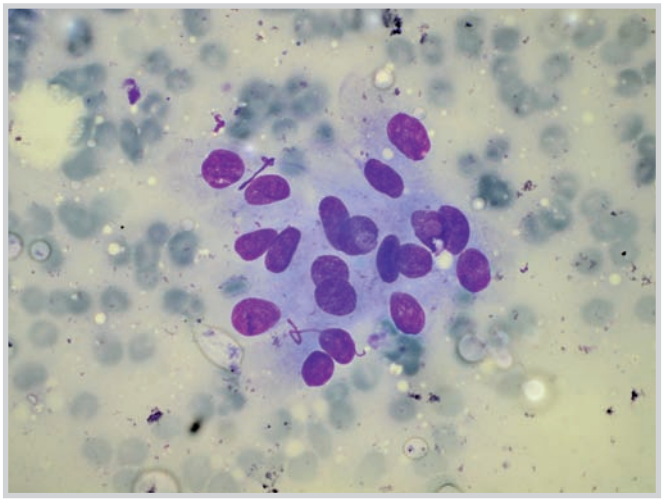

Fig. 24.22: Intranuclear inclusion in meningioma (MGG × HP)

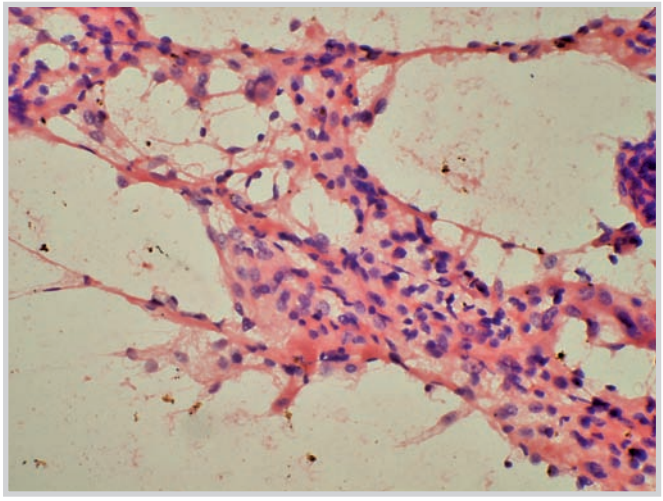

Fig. 24.23: Cells with moderate amount of cytoplasm with indistinct margin and monomorphic nuclei (H & E × MP)

BOX 24.8 **Meningioma**

- Tight clusters and dissociated cells
- Whorling appearance
- Oval to spindle cells with scanty cytoplasm
- Nuclear grooves and intranuclear inclusions
- Psammoma body

Differential Diagnosis

- Soft tissue tumor of spindle cell origin.

Olfactory Neuroblastoma[9]

This is also known as esthesioneuroblastoma. This tumor develops from the neuroepithelial cells in the olfactory mucosa.

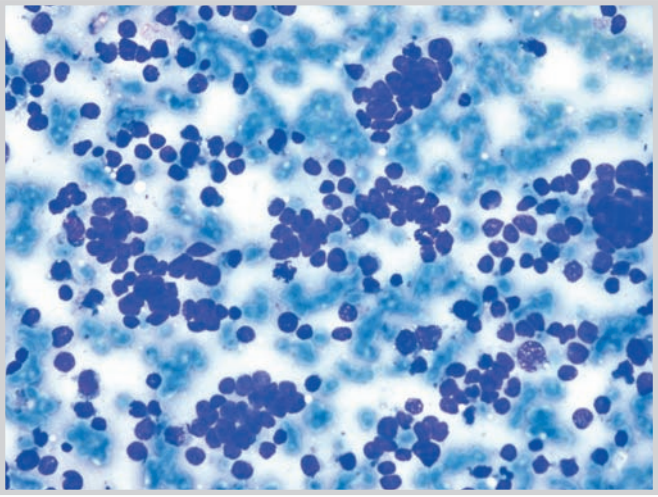

Fig. 24.24: Abundant discrete round to oval small cells with scanty cytoplasm in esthesioneuroblastoma (MGG × MP)

BOX 24.9 Olfactory neuroblastoma

- Discrete round to oval small cells
- Scanty deep blue cytoplasm
- Round monomorphic nuclei
- Indistinct to small nucleoli
- Fine to granular chromatin
- Homer-Wright rosettes

Olfactory neuroblastoma may occur in any age group. This tumor presents as soft mass in the roof of the nasal cavity.

Cytology (Fig. 24.24)

Fine needle aspiration cytology smears show abundant discrete round to oval small cells. The cells have scanty deep blue cytoplasm with round monomorphic nuclei. Nuclear contour of the cell is regular with indistinct to small nucleoli and fine to granular chromatin (**Box 24.9**). Homer-Wright rosettes are frequently seen in olfactory neuroblastoma. The background of the smear shows numerous naked nuclei.

ORBITAL LESIONS

Fine needle aspiration cytology provides a safe, simple and reliable outpatient department technique for the diagnosis of various orbital lesions.[10-16] FNAC is useful in nonresectable and retrobulbar lesions. FNAC is particularly helpful to diagnose the orbital recurrence or metastasis of a tumor because a definitive diagnosis can be obtained without any biopsy or surgical intervention. It also provides rapid diagnosis and avoids unnecessary surgical intervention in leukemia and lymphoma cases. The diagnostic accuracy of orbital FNAC ranges from 72% to 100%.[16-24] A low diagnostic accuracy rate was encountered mostly in the initial days of orbital FNAC. However, more recent and larger studies have shown consistently better results with an overall diagnostic accuracy of 100%.[16]

Techniques of Orbital Fine Needle Aspiration Cytology

In our institution, FNAC of orbital and eyelid lesion is always done under the supervision of an ophthalmic surgeon. Superficial and easily approachable lesions can be done directly without any image guidance. In case of deep-seated lesions, ultrasound or CT-guided FNAC is advisable. The patient should be in supine position. With the help of the ultrasonography (USG) probe the orbit is scanned to visualize the lesion. The eyeball is properly fixed by giving firm pressure with the probe. The patient is also requested to fix his eye during the procedure. Fine needle is introduced from the opposite side to the probe close to the bony wall of the orbit and the needle track is monitored continuously by means of USG visualization. The needle is moved to and fro within the lesion and gentle suction should be applied. The suction is released and the needle is withdrawn gently.[25] Multiple wet-fixed and air-dried smears are made and the material is also processed for the cell block preparation. If needed, a repeat FNAC could be done for the collection of material for ancillary investigations or from the residual material in the needle hub.

Complications

Fine needle aspiration cytology may give rise to various complications such as retrobulbar hemorrhage, hematoma, bleeding and infections. The more serious complications such as perforation of the globe and intracranial injury have also been described in literature.[16,17] In most of the time, conservative approach is enough to avoid any such complications. Extreme care should be taken to avoid the serious complications like the perforation of the globe.

Malignant Neoplasm of the Eyelid

Basal Cell Carcinoma

Basal cell carcinoma (BCC) is one of the commonest tumors of the eyelid. BCC represents almost 80–90% of all eyelid carcinomas.[26-28] These lesions are usually recognized by clinical examination and removed by surgical excision biopsy. Therefore, FNAC is rarely done in BCC.

Cytology

Detailed cytological features have been described in the skin section. The scrapping or FNAC smears of BCC show tight cohesive clusters of basaloid cells. Palisading-like nuclear arrangement at the periphery of the clusters is also noted. The tumor cells show round monomorphic nuclei with high nucleus-to-cytoplasm (N/C) ratio and uniformly distributed coarse chromatin.

Squamous Cell Carcinoma

Fine needle aspiration cytology smears of SCC show discrete and loosely cohesive clusters of keratinized epithelial cells in a necrotic background. The cells are polyhedral with cytoplasmic orangeophilia in Papanicolaou-stained smears. The nuclei show moderate degree of pleomorphism with hyperchromasia.

Sebaceous Carcinoma (Meibomian Carcinoma)

Sebaceous carcinoma commonly presents as eyelid mass. It has high morbidity and mortality and early diagnosis by FNAC is helpful for the management of the patient.

Cytology (Figs 24.25 and 24.26)

The FNAC smears show clusters and occasionally discrete malignant cells. The cells have moderate to abundant vacuolated cytoplasm and centrally placed moderately pleomorphic nuclei having coarse chromatin and prominent nucleoli. The malignant cells are often positive for oil red O.

Lesions of Lacrimal Gland

Pleomorphic Adenoma (Figs 24.27 and 24.28)

Pleomorphic adenoma is the commonest tumor of the lacrimal gland of the eye. The cytological features of pleomorphic adenoma are similar as noted in the salivary gland. FNAC smears show discrete and clusters of monomorphic round to oval cells in a chondromyxoid background. The cells have moderate amount of cytoplasm and central to eccentric nuclei. Clusters and discrete spindle-shaped myoepithelial cells are also noted within the chondromyxoid stromal material.

Differential Diagnosis

Adenoid cystic carcinoma (ACC).

Adenoid Cystic Carcinoma

Both solid and cribriform types of ACC are seen on FNAC. Solid variant of ACC shows predominantly small monomorphic round cells. The cytoplasm of the cell is scanty. Nuclei are hyperchromatic and monomorphic. The smears are usually devoid of any typical pink globules.

In the cribriform variant, the smears show large, spherical globules of material surrounded by tumor cells. The individual cell morphology is similar to that of solid variant.

Mucoepidermoid Carcinoma

Fine needle aspiration cytology smears of mucoepidermoid carcinoma show discrete malignant cells. The cells contain abundant clear cytoplasm with central moderately pleomorphic nuclei. Occasional squamoid cells are also noted.

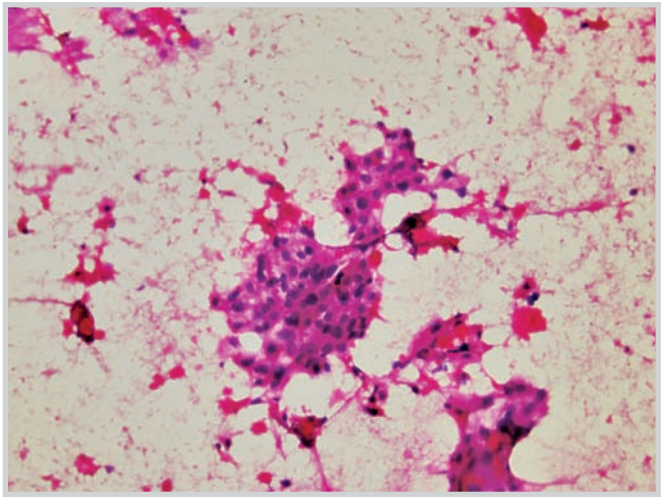

Fig. 24.25: Clusters of malignant cells in meibomian carcinoma (H & E × MP)

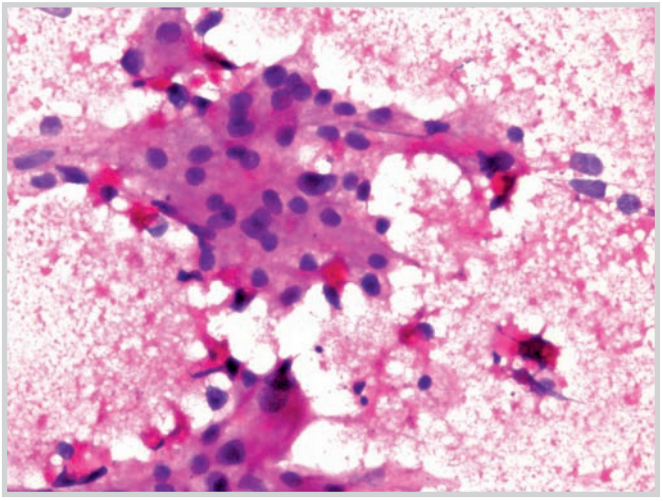

Fig. 24.26: Cells with abundant cytoplasm and centrally placed mildly pleomorphic nuclei in meibomian carcinoma (H & E × HP)

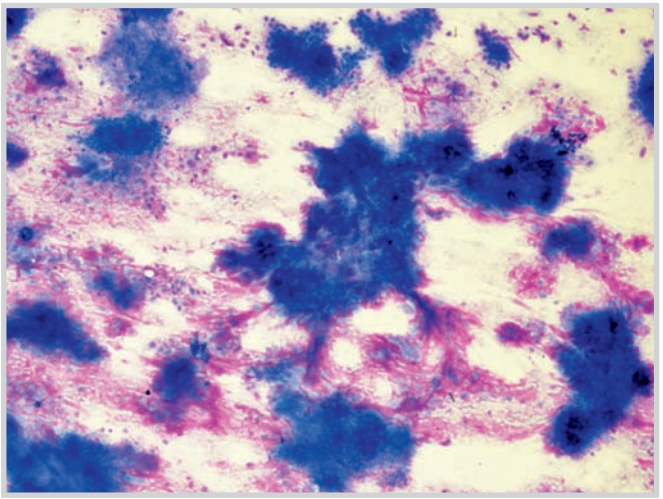

Fig. 24.27: Discrete and clusters of monomorphic round to oval cells in a chondromyxoid background in pleomorphic adenoma of lacrimal gland (MGG × LP)

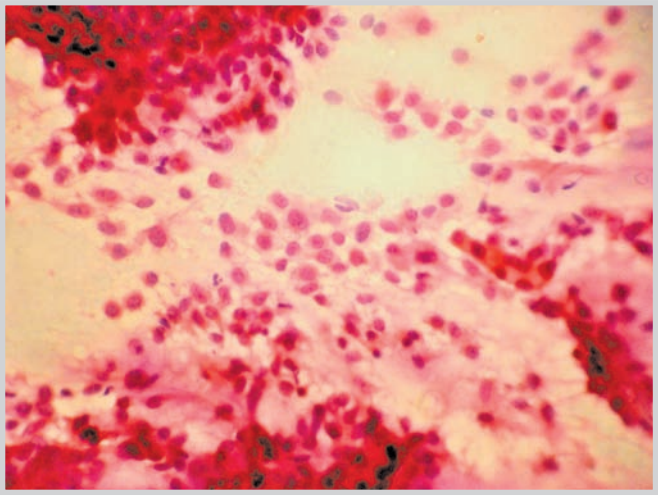

Fig. 24.28: Monomorphic round to oval cells with moderate amount of cytoplasm in pleomorphic adenoma of lacrimal gland (H & E × MP)

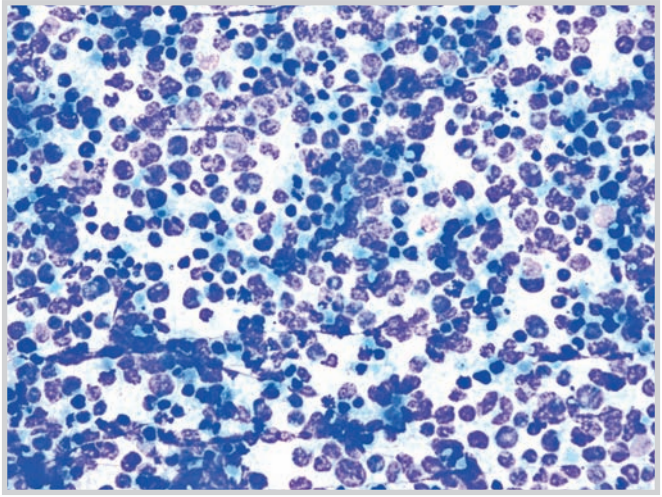

Fig. 24.29: Predominantly discrete population of small round cells in retinoblastoma (MGG × MP)

Intraorbital Tumors

Retinoblastoma

Retinoblastoma is the commonest intraocular malignancy in children. Usually, clinical presentation and indirect ophthalmoscopic examination are sufficient for the diagnosis of retinoblastoma. FNAC of the retinoblastoma is done only in doubtful cases. The patient usually presents with progressive deterioration of vision or proptosis.

Cytology (Figs 24.29 and 24.30)

Fine needle aspiration cytology smears show predominantly discrete population of small round cells. Occasional rosette-like structures may also be noted. Flexner-Wintersteiner rosettes and Homer-Wright rosettes in FNAC smears have been described in the literature.[21,29] Flexner-Wintersteiner rosettes are seen as cells around the lumen, whereas, in case of Homer-Wright rosettes, the cells are arranged around cobweb-like material.

The tumor cells are small round with scanty cytoplasm. The nuclei are round, monomorphic and hyperchromatic with fine nuclear chromatin (**Box 24.10**). Occasional prominent nucleoli are also seen. Tight cohesion and nuclear molding among the tumor cells are the distinct cytological features of retinoblastoma.[29-32]

Retinoblastoma should be differentiated from various small round cell tumors, such as lymphoma or leukemia and metastatic neuroblastoma involving the orbit. Immunocytochemistry is helpful in the differential diagnosis of small blue round cell tumors.[26,31]

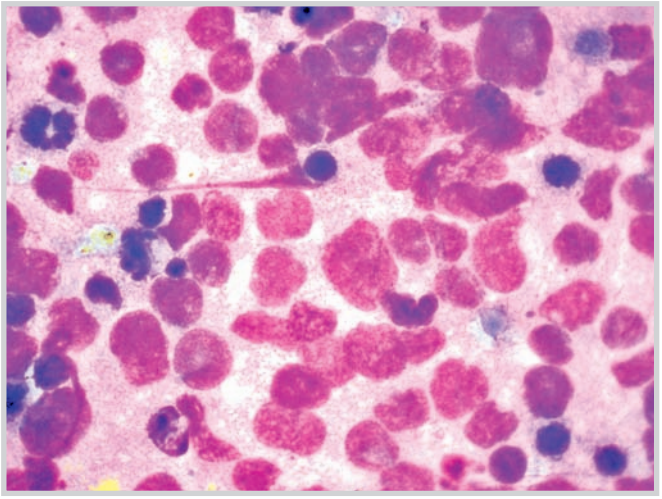

Fig. 24.30: Round, monomorphic cells with fine nuclear chromatin and scanty cytoplasm in retinoblastoma [MGG × oil immersion (OI)]

BOX 24.10 Retinoblastoma

- Small round to oval cells in clusters and rosettes
- Round cells
- Scanty cytoplasm
- Hyperchromatic nuclei
- Inconspicuous nucleoli

Rhabdomyosarcoma

Rhabdomyosarcoma is the most common malignant mesenchymal neoplasm of the orbit in children. FNAC smears show clusters, and single cells. The tumor cells are small and round with moderate amount of cytoplasm. The nuclei are round, central to eccentric in position with fine nuclear chromatin and prominent nucleoli. Frequent bi- and multinucleation are also seen.

Rhabdomyosarcoma is positive for desmin, MyoDl and myogenin. Expression of these antigens is helpful to distinguish this tumor from retinoblastoma and neuroblastoma.[33]

Malignant Melanoma

Malignant melanoma of the orbit develops from the uveal tract. FNAC smears of the orbital melanoma show discrete and small clusters of cells. The cells show moderate amount of cytoplasm and moderately pleomorphic nuclei. Large prominent macronucleoli is often seen in melanoma cases. Majority of malignant melanoma cases show intracytoplasmic melanin pigment. In case of amelanotic melanoma, immunocytochemistry particularly HMB-45 is helpful.

Lymphoma

Primary orbital lymphomas account for one-tenth of all orbital tumors. About 1.3% of the cases of systemic lymphomas may also secondarily affect the orbit.[10,34,] Majority of the orbital lymphomas are well-differentiated lymphocytic, lymphoplasmacytoid or centrocytic or centroblastic lymphomas of the extranodal marginal type.[35] Burkitt's lymphomas also involve the orbit. The cytology smears of the lymphoma depend on the type of NHL (**Figs 24.31** to **24.33**).

Detailed immunocytochemistry and flow cytometric immunophenotyping are helpful in the diagnosis and subtyping of lymphomas.[36]

Leukemic Infiltration

The patients with leukemic infiltration of the orbit may often present as proptosis. The smears show numerous blasts along with myelo- and metamyelocytes. Auer rods can be demonstrated in occasional myeloblasts.

Meningiomas

Meningiomas are benign tumors derived from arachnoid cells of the meninges. Meningiomas are the most common benign neoplasms of the orbit and the patients usually present with proptosis and progressive loss of eyesight. The cytological features are same as described in the previous section of this chapter.

Optic Glioma (Figs 24.34 and 24.35)

Optic glioma presents as retro-orbital space occupying lesion. The cytology smear of optic glioma shows oval to spindle-shaped cells embedded in pink fibrillary material.

Metastatic Malignancy[22]

A large variety of malignancies may metastasize in the orbit. The common primary sites are head and neck carcinomas, lung, breast and colon. Cytological features of such metastatic masses depend on the exact type of primary carcinomas.

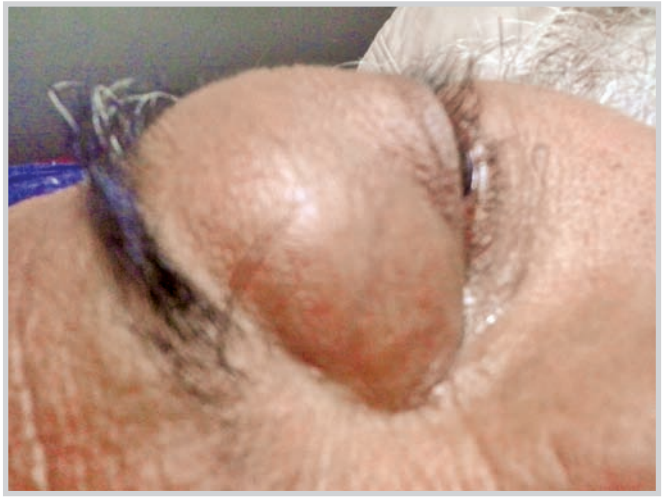

Fig. 24.31: Slow growing intraorbital swelling in a case of orbital lymphoma

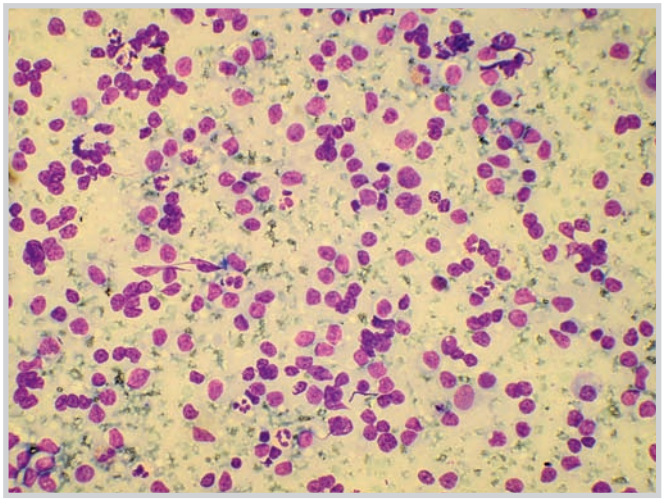

Fig. 24.32: Round discrete monomorphic cells in mucosa-associated lymphoid tissue lymphoma (MALTOMA) of orbit (MGG × MP)

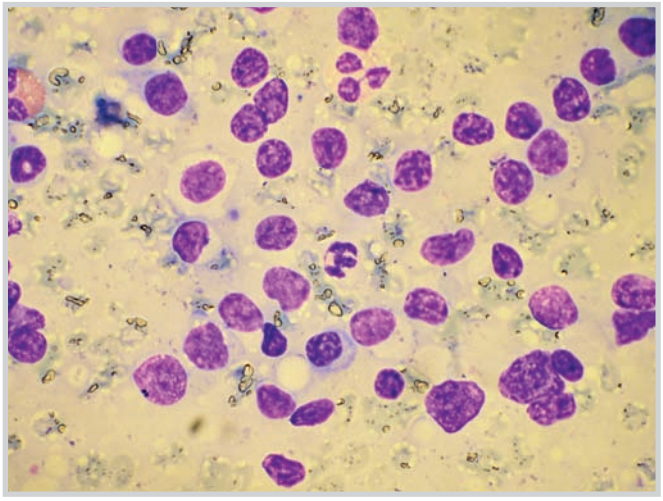

Fig. 24.33: Round cells with scanty cytoplasm and monomorphic nuclei with clumped chromatin MALTOMA of orbit [MGG× oil immersion (OI)]

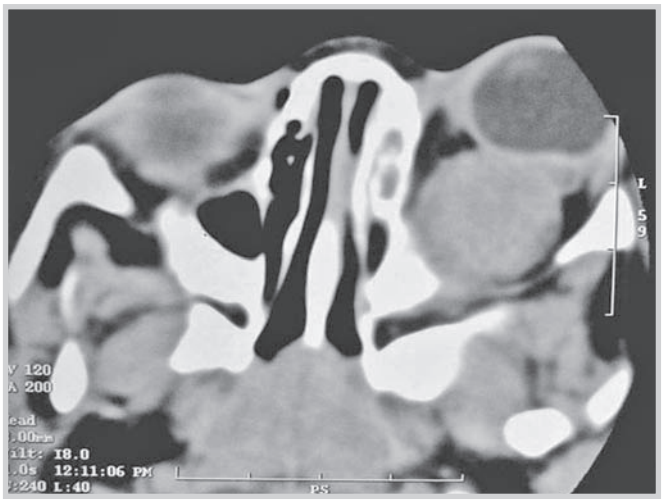

Fig. 24.34: Computed tomographic scanning photograph of optic glioma (*Source*: Dr Anupam Lal, Additional Professor, Department of Radiodiagnosis, PGIMER, Chandigarh, India)

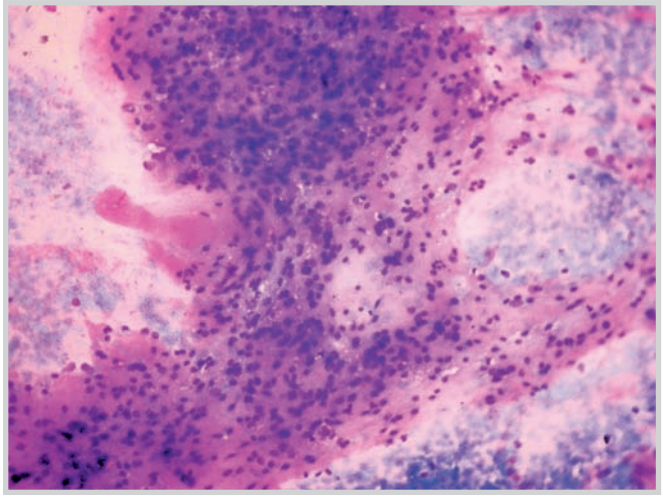

Fig. 24.35: Oval to elongated cells embedded in pink fibrillary background (MGG × MP)

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CHAPTER 25

Salivary Gland

Chapter Contents

ANATOMY AND HISTOLOGY OF THE SALIVARY GLAND

Salivary glands are the exocrine glands that are responsible for secretion of the saliva. There are two groups of salivary glands:

1. *Major salivary glands*: Major salivary glands consist of pair of parotid glands, submandibular glands and sublingual salivary glands.
2. *Minor salivary glands*: There are large number of minor salivary glands in the mucosa of the oral cavity, nasal sinuses, larynx and bronchial tree.

Parotid glands are mainly serous glands. Each gland weighs about 15–25 gm. The parotid gland is situated in the angle of mandible. Anatomically, the gland is bounded anteriorly by masseter muscle, superiorly by zygomatic arch, posteriorly by the external auditory canal, and inferiorly by styloid process, internal carotid artery and jugular vein. The Stensen's duct is the main excretory duct of the parotid gland. This is about 6 cm in length and 5 mm in diameter. The duct travels along the masseter muscle and then pierces through the buccinator muscle to open near the upper second molar tooth within the oral cavity. During the embryological development of the parotid gland, there is incorporation of lymphoid tissue within it and that may be the source of Warthin's tumor. The gland is divided into superficial and deep compartment and facial nerve with its branches passing in between these two compartments. Majority of the tumor arises from the superficial lobe of the parotid gland and form superficial bulging whereas the tumors of the deeper lobe of the parotid gland present as parapharyngeal swelling. Therefore, fine needle aspiration cytology (FNAC) of the parotid gland tumor is easy to perform and the subsequent surgical resection of the tumor is not difficult.

Submandibular salivary gland is located in the submandibular triangle which is bounded anteriorly by anterior belly of digastrics, posteriorly by posterior belly of digastrics and superiorly by mandible. This is a seromucinous gland and weighs 7–8 gm. The gland has two lobes: (1) superficial and (2) deep lobes. The secretion of the gland flows through the Wharton's duct that opens into the floor of the mouth.

The sublingual gland is the smallest salivary gland and weighs only 3 gm. This is a mucinous gland. The gland is located in the sublingual fossa and the secretion of the gland flows through multiple excretory ducts to open into the oral cavity.

Histology

The major salivary gland is composed of secretory part and duct part. The secretory part is made of acini which are lined by serous, mucinous or mixed (seromucinous) type of cells. The cells in acini are arranged as lobules encircled by myoepithelial cells. Each acini opens into the intercalated ducts that further joins with each other and forms larger excretory duct.

INDICATIONS OF FINE NEEDLE ASPIRATION CYTOLOGY OF THE SALIVARY GLANDS

Fine needle aspiration cytology is indicated in any space occupying lesion of the salivary glands. The patients usually present with the complaints of swelling, pain, facial nerve palsy, palatal palsy and paresthesia. FNAC of salivary gland is especially helpful as incision biopsy may lead to fistula formation, potential infection in the plane of surgery and facial nerve palsy. Besides that, FNAC has high sensitivity and specificity. It is a rapid and safe technique and devoid of any serious complications.[1-8]

Fine needle aspiration cytology helps to differentiate a neoplastic from a non-neoplastic lesion with confidence (**Box 25.1**). In case of non-neoplastic inflammatory lesion, a conservative treatment may be helpful. Moreover, the aspirated material can be sent to microbiological or other ancillary tests. FNAC diagnosis of the neoplastic lesion helps the surgeon to plan surgery according to the nature of the tumor.

Contraindications

There are no absolute contraindications of FNAC of the salivary gland. However, it is better to avoid FNAC in case of bleeding diathesis (**Box 25.2**). FNAC should not be done in absence of any swelling of the salivary gland or if there is infection over the skin of the gland.

BOX 25.1 **Indications and advantages of fine needle aspiration cytology (FNAC)**

Indications: Any palpable swelling of the salivary gland.
Advantages:
- Rapid
- Safe
- High sensitivity and specificity
- Cheap
- Avoids any unnecessary surgery
- Helps in planning of surgery in case of neoplasm
- Various ancillary investigations can be done: Microbiological examination, flow cytometry, cell block

BOX 25.2 **Complications and contraindications**

Complications:
- Hemorrhage
- Facial pain
- Infection

Contraindications:
- Bleeding diathesis
- Severe skin infection
- No swelling

Complications

There is no serious complication reported after FNAC of the salivary gland. The needle tract seeding of the malignant cell is a myth.[9]

Rarely there may be local hemorrhage, short-term facial nerve pain, or infection. Careful FNAC procedure such as local compression after FNAC avoids local hemorrhage. Similarly, strict maintenance of sterility avoids any possibility of infection.

Fine Needle Aspiration Cytology: Technical Consideration

Fine needle aspiration cytology of the salivary gland is done preferably by thin needle with 23 gauze diameter. It should be done from multiple sites to have adequate sampling (**Box 25.3**). Cystic lesions are the potential source of mistakes. Therefore, in case of cystic lesion a repeat FNAC should be done from the residual swelling after drainage of the cyst fluid. The repeat FNAC may help to avoid missing of the neoplasm with cystic changes. Gross appearance of the aspirated material may also provide important information. Aspiration of thick gelatinous material often indicates pleomorphic adenoma (PA). Drops of dirty fluid are aspirated in case of Warthin's tumor. Aspiration of pus like material usually indicates suppurative inflammation. In every FNAC, both May-Grünwald-Giemsa (MGG) and Papanicolaou's stains should be done. MGG stain is the metachromatic stain and it is helpful to demonstrate stromal material. This is particularly useful in PA and adenoid cystic carcinoma (ACC). Papanicolaou's stain is particularly helpful to demonstrate the squamoid component of the mucoepidermoid carcinoma (MEC). In addition, depending on the situation the material should be kept for gram stain and microbiological culture. There is limited scope of immunocytochemistry in case of salivary gland tumors. However, cell block may be helpful for cytochemistry and histological features.

BOX 25.3 **Technical and clinical considerations**

Technical:
- FNAC from multiple sites
- Cyst should be reaspirated after drainage of fluid
- MGG and Papanicolaou's stain to do
- Tests for ancillary investigations: Microbial culture, FCM, cell block

Clinical indications of malignancy:
- Rapid enlargement
- Swelling fixed into skin or deeper structure
- Facial nerve palsy
- Pain in the ear

Abbreviations: FNAC, fine needle aspiration cytology; MGG, May-Grünwald-Giemsa; FCM, flow cytometric

Clinical Evaluation

Detailed clinical history is helpful in demonstrating the nature and exact type of the lesion. The triads of pain, fever and local sign of inflammation indicate an inflammatory lesion. Most of the salivary gland tumor particularly low-grade cancer presents with a slow growing painless mass. Clinically, there may not be any distinctive features between a benign and a malignant tumor. However, a rapidly increasing mass, facial palsy, paresthesia, fixation of the swelling with adjacent skin and deeper structure and lymph nodal enlargement indicate the malignant nature of the lesion.

Overview of the Diagnostic Challenges

Fine needle aspiration cytology diagnosis of the salivary gland tumors often poses diagnostic challenges to the cytologist. The various facets of the diagnostic problems are:

- *Varied and heterogeneous nature of salivary gland neoplasia*: Large varieties of the epithelial and nonepithelial tumors have been described by World Health Organization (WHO) in salivary gland.[10] The cell of origin of these tumors is common and they differ only by architectural pattern. Therefore, it is difficult to diagnose them with certainty on FNAC.
- *Cystic lesions (***Box 25.4***)*: Many non-neoplastic and neoplastic lesions of salivary gland are cystic. The non-neoplastic cysts may be due to obstruction of the excretory ducts of the salivary gland (retention cyst), lymphoepithelial cyst or salivary duct cyst. At times, branchial cyst may also be present in upper later neck simulating salivary gland lesion. Many salivary gland neoplasms may show cystic changes such as Warthin tumor, MEC, PA and cyst adenocarcinoma. Inadequate sampling from the cystic area of such tumors is the potential source of false negative diagnosis.
- *Low-grade tumor*: Many salivary gland tumors are low grade and therefore difficult to distinguish from the normal aspirate and vice versa. Abundant benign salivary acinar cells along with background of stripped nuclei of the acinar cells may often simulate acinic cell carcinoma. Similarly, low-grade mucoepidermoid tumor may be misdiagnosed as benign lesion.
- *Benign versus malignant tumor of the same type of tumor*: At times, it is very difficult to distinguish the benign and the malignant counterpart of a tumor. Such as myoepithelioma is difficult to distinguish from malignant myoepithelioma. Similarly, basal cell adenoma (BCA) is difficult to distinguish from basal cell adenocarcinoma.
- *Lymphoid rich lesion (***Box 25.5***)*: Lymphoid cell rich aspirate of the salivary gland lesion may also pose diagnostic challenge. Aspirated material rich in lymphoid cells may be noted in benign non-neoplastic conditions such as chronic sialadenitis, lymphoepithelial lesions, and granulomatous sialadenitis. Neoplastic conditions such as Warthin's tumor, MEC and acinic cell tumor may show good amount of lymphoid cells. At times, it is very difficult to differentiate a low-grade non-Hodgkin's lymphoma (NHL) from the lymphoepithelial lesions. Demonstration of monoclonality with the help of flow cytometry (FCM) may be helpful in such conditions.
- *Squamoid differentiation*: Fine needle aspiration cytology of the salivary gland may often show epithelial cells with squamoid differentiation. Metaplastic benign squamous cells are seen in chronic sialadenitis, Warthin's tumor and PA. The individual cells do not show any nuclear enlargement and pleomorphism and therefore they usually do not create any problem. However, inadequate sample containing only the metaplastic squamous cells may be mistaken as low-grade MEC. Malignant squamous cells are seen in MEC, and primary or metastatic squamous cell carcinoma of the salivary gland.
- *Oncocytic changes*: Many neoplastic and non-neoplastic lesions of the salivary gland may show oncocytic changes and thereby may create diagnostic confusion. Benign tumors that commonly display oncocytic changes are oncocytoma and Warthin's tumor. Whereas, the malignant tumor with oncocytic changes are oncocytic carcinoma and MEC. The presence of lymphoid cells in Warthin's tumor usually helps to differentiate this tumor from oncocytoma.

BOX 25.4 **Cystic lesions in the salivary gland**

Non-neoplastic cyst:
- Retention cyst due to obstruction of the salivary duct
- Lymphoepithelial cyst
- Salivary duct cyst

Comments: Does not pose much problem except nuclear atypia due to inflammation

Neoplastic cyst:
- Warthin's tumor
- Mucoepidermoid carcinoma
- Pleomorphic adenoma
- Cyst adenocarcinoma

Comments: Inadequate sampling may cause false negative diagnosis

NORMAL SALIVARY GLAND CELLS

Fine needle aspiration cytology of the normal salivary gland usually yields scanty material.

BOX 25.5 **Lymphoid rich lesions**

Non-neoplastic:
- Chronic sialadenitis
- Lymphoepithelial lesions
- Granulomatous sialadenitis

Neoplastic:
- Lymphoma
- Warthin's tumor
- Mucoepdermoid carcinoma
- Acinic cell tumor

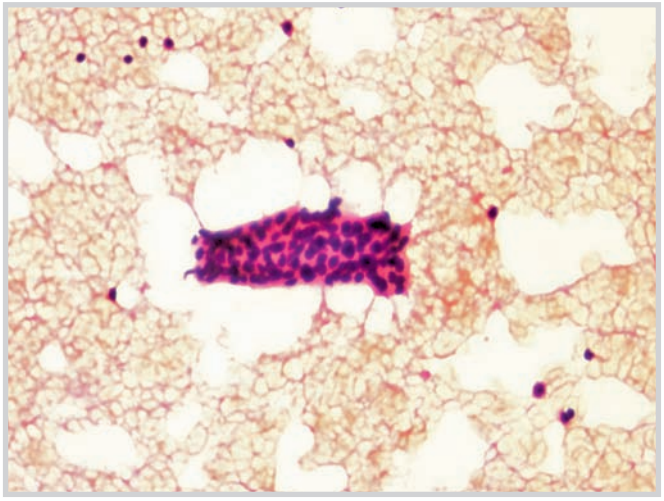

Fig. 25.1: Round to oval monomorphic benign ductal cells [May-Grünwald-Giemsa (MGG) stain × medium power (MP)]

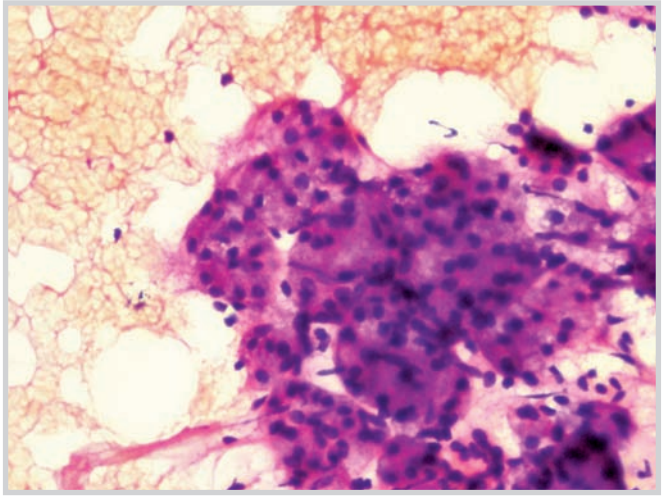

Fig. 25.2: Loose grape like cluster of benign salivary acinar cells [hematoxylin and eosin (H & E) stain × MP)

- *Benign ductal cells* (**Fig. 25.1**): Ductal cells are usually present in tight small clusters or honeycombed like monolayered sheets. The individual cells are round with scanty cytoplasm having monomorphic round nuclei.
- *Acinar cells* (**Fig. 25.2**): These cells are commonly present as loose grape-like clusters or discretely. The individual cells are pyramid in shape with abundant foamy cytoplasm and small eccentrically placed round nuclei. Due to cytoplasmic fragility, the acinar cells often present as bare nuclei and these cells should be distinguished from the background lymphocytes.
- *Myoepithelial cells*: Myoepithelial cells are oval to spindle-shaped with scanty indistinct cytoplasm. Oval plasmacytoid myoepithelial cells may also be seen.

BOX 25.6 Retention cyst

- Watery or mucoid fluid
- Macrophages
- Cuboidal, columnar or squamous cells
- Polymorphs and lymphocytes

Other Components

Fragments of fibrous and adipose tissue may also be seen in the background of the normal salivary aspirate. Due to the presence of intraparotid lymph node, lymphoid cells are often noted in the normal salivary aspirate.

It is important to note that FNAC smears containing only normal salivary gland cells in a swelling is mainly caused by sampling error. The common causes of finding normal salivary gland cells in FNAC smears are:

- Sampling error in a neoplastic lesion
- Sialadenitis
- sialolithiasis
- Idiopathic enlargement of parotid gland and
- Acinic cell carcinoma mistaken as normal acinar cells. Therefore, in case of any suspicion, a repeat FNAC should always be performed.

SALIVARY GLAND LESIONS

Non-Neoplastic Conditions:

- *Cystic lesions of the salivary gland*: Approximately 5% of the salivary gland lesions are cystic in nature.[11] **Box 25.4** highlights different types of cystic lesions of the salivary gland.
- *Retention cyst* (**Box 25.6**): The deep seated retention cysts are firm on feel and may be mistaken as neoplastic lesion. The retention cyst develops due to obstruction of the salivary duct. FNAC yields clear watery to mucoid fluid. Smears from the fluid show macrophages along with degenerated epithelial cells. The epithelial cells may be cuboidal, columnar or squamous. The cysts may show variable number of polymorphs and lymphocytes depending on superadded infection.
- *Simple cyst*: These are mostly superficial and clinically recognizable as benign lesion. FNAC smear usually shows scanty benign squamous cells.
- *Mucocele*: This is due to the extravasations of the mucus filled cyst of the minor salivary gland to the adjacent soft tissue. FNAC smears show histiocytes in the background of mucinous material.
- *Lymphoepithelial cyst* (**Box 25.7**): Lymphoepithelial cysts are seen in adult. These cysts are unilateral and mainly involve the parotid gland. Lymphoepithelial cysts are also noted in human immunodeficiency virus (HIV) patients.[12] HIV related lymphoepithelial cysts are usually bilateral.

The cysts are lined by squamous epithelium with underneath lymphoid cells. FNAC of the cyst shows foamy macrophages, reactive lymphocytes along with squamous cells. Multinucleated giant cells may also be seen.

BOX 25.7 Lymphoepithelial cyst

- Foamy macrophages
- Reactive lymphocytes
- Squamous cells
- Multinucleated giant cells

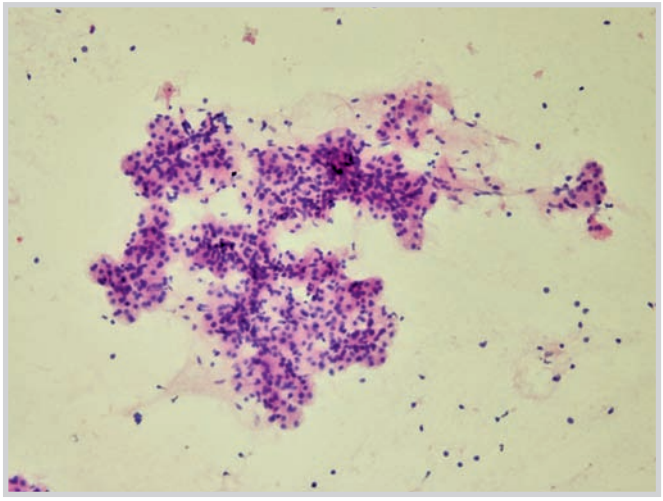

Fig. 25.3: Abundant clusters of benign salivary acini in sialadenosis [H & E stain × low power (LP)]

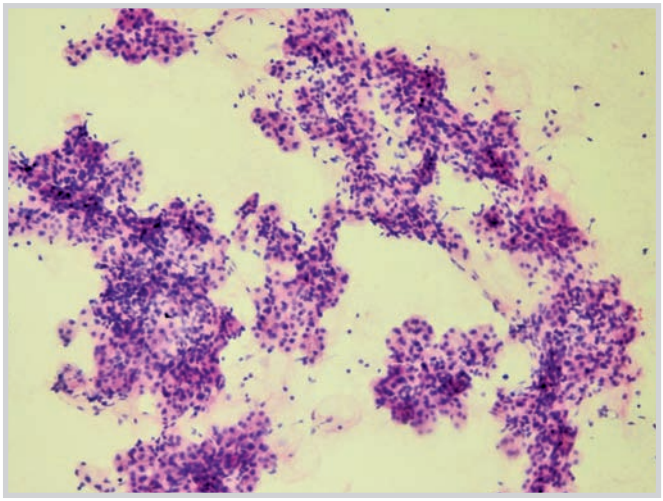

Fig. 25.4: Large number of salivary acinar cells in sialadenosis (H & E stain × MP)

Sialadenosis

Sialdenosis is an uncommon entity. It is a non-neoplastic and noninflammatory lesion of the salivary glands. It usually involves bilateral parotid gland. The swelling is diffuse and doughy in feel. Sialdenosis is commonly associated with diabetes mellitus, obesity, chronic alcohol intake and cirrhosis.

Cytology (Figs 25.3 and 25.4)

Fine needle aspiration cytology smears show scattered clusters of ductal and abundant acinar cells. Background of the smear is free from lymphocytes. The acinar cell may show mild nuclear enlargement. In long standing case, the gland becomes atrophic and is replaced with fibrofatty tissue and FNAC may show scanty cellularity.

Sialadenitis

Acute Sialadenitis

Acute sialadenitis usually occurs in older patients, postoperative patients with poor oral hygiene, sialolithiasis or in case of obstruction of the salivary duct. In younger patient and child, it may occur due to viral infection such as mumps. The patient of acute sialadenitis presents with pain, swelling of the salivary gland and fever. Both parotid and submandibular gland may be involved by acute sialadenitis. However, parotid gland involvement is more common because serous secretion of this gland has less antimicrobial property. The underlying factors in any acute sialadenitis are poor oral hygiene, dehydration and immunodeficiency.

Cytology

Fine needle aspiration cytology smears show abundant polymorphs, and necrotic cellular debris. In addition, many clusters and dissociated benign ductular and acinar cells are seen.

Chronic Sialadenitis[13-15]

The cases of chronic sialadenitis usually presents with recurrent enlargement of the salivary gland along with pain during chewing food. This is usually due to obstruction of the ducts of salivary glands by stone. The proximal to the obstruction of the glands may be affected by infection. Near about 80% of chronic sialadenitis occurs in the parotid gland and the rest 20% occurs in the submandibular gland. The sublingual gland is infrequently involved by chronic sialadenitis.

Cytology (Figs 25.5 and 25.6)

Fine needle aspiration cytology smears show many clusters of ductal cells, scanty acinar cells and lymphocytes in a background of mucinous material. The sparse amount of acinar cells is usually due to chronic destruction of such cells.

Lymphoepithelial Sialadenitis[15,16]

This disease is also known as benign lymphoepithelial lesion and Mikulicz's disease and myoepithelial sialadenitis. This is an autoimmune condition and no age is exempted. The patients are usually female with unilateral or bilateral enlargement of the parotid gland. The submandibular gland is uncommonly involved. In 50% of the cases, lymphoepithelial sialadenitis (LESA) is associated with Sjögren's syndrome. The patients with LESA have higher risk to develop NHL particularly B cell type. Therefore, the cases of LESA should always be followed up by FNAC.

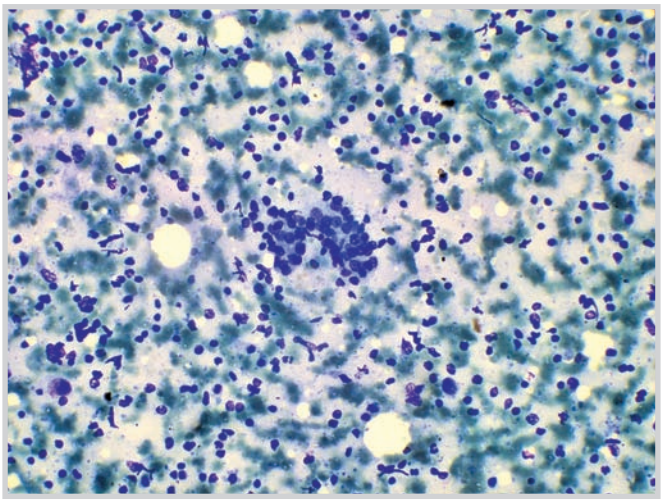

Fig. 25.5: Abundant lymphocytes and ductal cells in chronic sialadenitis (MGG stain × MP)

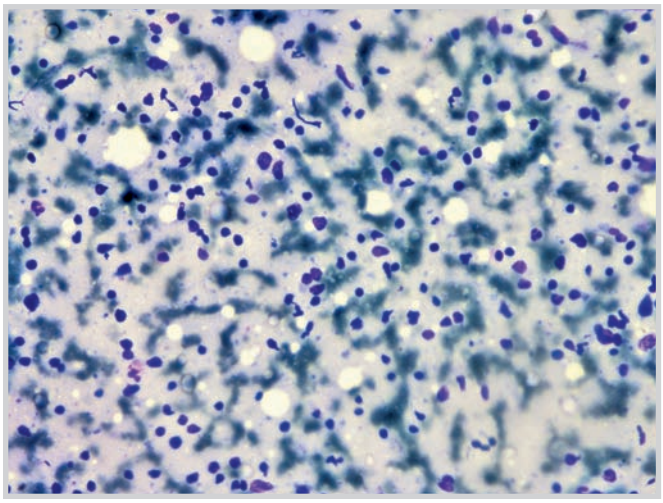

Fig. 25.7: Abundant reactive lymphoid cells in lymphoepithelial sialadenitis (MGG stain × MP)

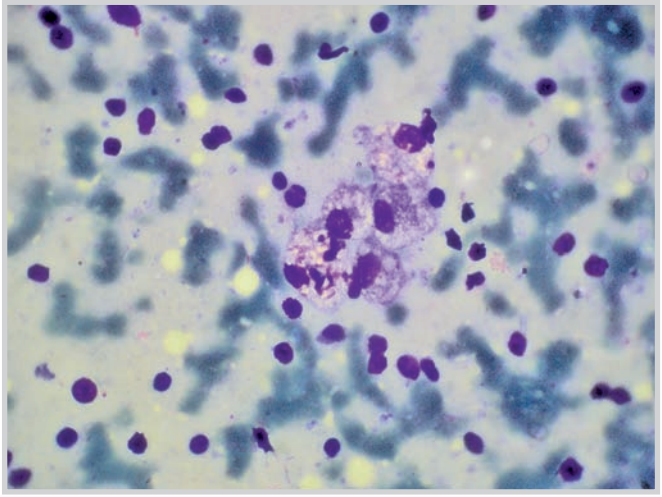

Fig. 25.6: Foamy macrophages and lymphocytes in chronic sialadenitis (MGG stain × MP)

BOX 25.8 Lymphoepithelial sialadenitis

- Mature lymphocytes and follicular center cells
- Ductal cells

Cytology (Fig. 25.7)

The cytology smears show abundant reactive lymphoid cells consisting of mature lymphocytes, centroblasts, follicular center cells and plasma cells (**Box 25.8**). In addition, occasional loose clusters of ductal epithelial cells along with lymphocytic infiltrate may also be seen. It is necessary to exclude the possibility of a low-grade NHL.

Differential Diagnosis

- *Lymphoma*: Low-grade mucosa associated lymphoma is often difficult to distinguish from the LESA. Monomorphic population of immature lymphoid cells is noted in lymphoma, whereas in case of LESA the lymphoid cell population is polymorphic. Demonstration of monoclonal nature of the lymphoid cells may be necessary for the diagnosis of low-grade NHL.
- *Warthin tumor*: This may also show large amount of reactive lymphoid cells and may simulate LESA. However, Warthin tumor shows good number of oncocytic cells.
- *Lymphoepithelial cyst*: This may yield fluid with predominant lymphocytes.
- *Intraparotid reactive lymph node*: This may also show identical cytological features.
- *Chronic sialadenitis*: This may pose diagnostic difficulty because of similar cytological features. However, the amount of reactive lymphoid cells is usually less in chronic sialadenitis.

Granulomatous Sialadenitis

Granulomatous sialadenitis is caused by a large variety of diseases such as tuberculosis, sarcoidosis, fungal infections and duct obstruction due to stone.[17]

Fine needle aspiration cytology smears show epithelioid cell granulomas, multinucleated giant cells, lymphocytes and histiocytes. In addition, salivary ductal cells may be seen. Epithelioid cells should not be mistaken as component of epithelial tumor. Ziehl-Neelsen stain should always be done to exclude the possibility of tuberculosis.

NEOPLASTIC LESIONS

World Health Organization has classified salivary gland lesions into epithelial (benign and malignant), soft tissue tumors, hematolymphoid tumors and metastatic tumors[10] (**Table 25.1**).

TABLE 25.1: Classification of salivary gland tumors [modified from World Health Organization (WHO) classification][10]

Benign epithelial tumor	*Malignant epithelial tumor*	*Soft tissue tumors*	*Hematolymp-hoid tumor*	*Metastatic tumor*
• Pleomorphic adenoma • Myoepithelioma • Basal cell adenoma • Warthin tumor • Oncocytoma • Canalicular adenoma • Lymphadenoma • Duct papilloma	• Adenoid cystic carcinoma • Mucoepidermoid carcinoma • Acinic cell carcinoma • Epithelial myoepithelial carcinoma • Polymorphous low-grade adenocarcinoma • Basal cell adenocarcinoma • Squamous cell carcinoma • Carcinoma ex pleomorphic adenoma • Adenocarcinoma	• Hemangioma	• Extranodal marginal zone lymphoma • Diffuse large B cell lymphoma • Hodgkin lymphoma	• Metastatic tumor

Benign Neoplasm

Pleomorphic Adenoma[18-20]

Pleomorphic adenoma is the most common tumor of the salivary gland and constitutes about 60% of all tumors of the salivary gland. About 80% cases of PA develop in parotid gland, 10% in submandibular gland and the rest 10% cases in the paranasal sinuses, oral cavity, upper respiratory tract and skin.[21] PA commonly presents as a solitary well-defined, slow growing and painless swelling. Mean age of the patient is around 46 years and females are commonly affected than male.[21] About 90% of PA in parotid gland arises in the superficial lobe of the gland and small number of cases of PA may develop in the deep lobe of the parotid gland medial to the facial nerve. Tail of the parotid gland near the angle of the mandible is often involved by PA and may simulate as lymph nodal swelling.

Pleomorphic adenoma is also known as mixed tumor as it contains both epithelial and also mesenchymal or stromal material. The tumor possibly develops from the pluripotent reserve cells of the intercalated duct.

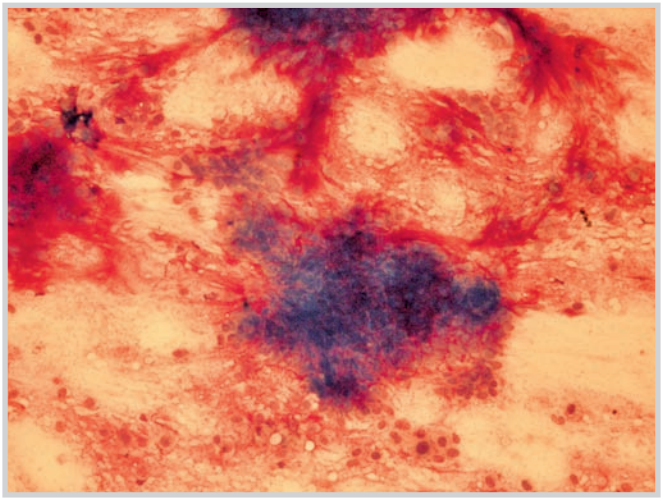

Fig. 25.8: Discrete and clusters of cells embedded in magenta colored chondromyxoid material in pleomorphic adenoma (MGG stain × LP)

Cytology (Figs 25.8 to 25.12)

Fine needle aspiration cytology of the PA yields thick mucoid like material that is often difficult to spread. MGG smears show abundant deep magenta colored fibrillar chondromyxoid stromal material (**Box 25.9**). This stromal material is pale grayish color in Papanicolaou's stain and difficult to recognize. Therefore, MGG stain is mandatory for proper recognition of PA as this demonstrates the detailed character of stromal material. The epithelial cells are present predominantly in cohesive clusters with honeycomb like pattern and also singly. The individual cells are round to oval with moderate to scanty cytoplasm having distinct cell border. Myoepithelial cells of PA are usually embedded in the myxoid stroma. These cells are oval with moderate cytoplasm and eccentric nuclei simulating plasma cells. Other than the plasmacytoid appearance, myoepithelial cells may be spindle shaped polygonal, epithelioid or clear appearance. The proportion of cellular and stromal component is extremely variable in PA. At times, epithelial component may form the main tumor mass and the smears may show predominantly cellular component. This variety of cellular PA has no separate prognostic importance. However, cellular PA may pose diagnostic problem. On contrary, some cases of PA

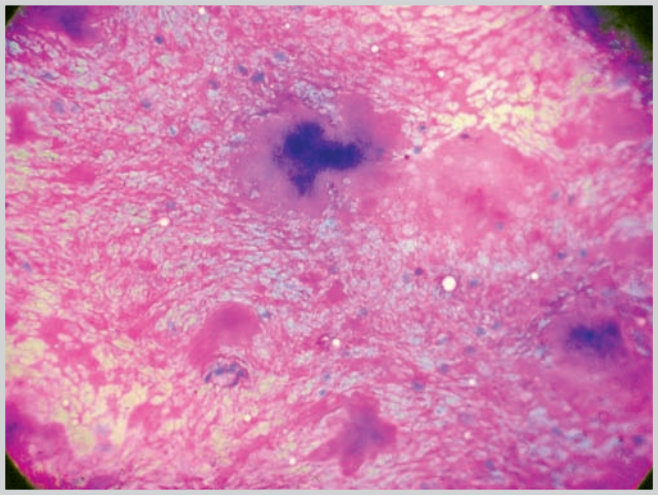

Fig. 25.9: Fibrilary magenta colored chondromyxoid material in pleomorphic adenoma (MGG stain × LP)

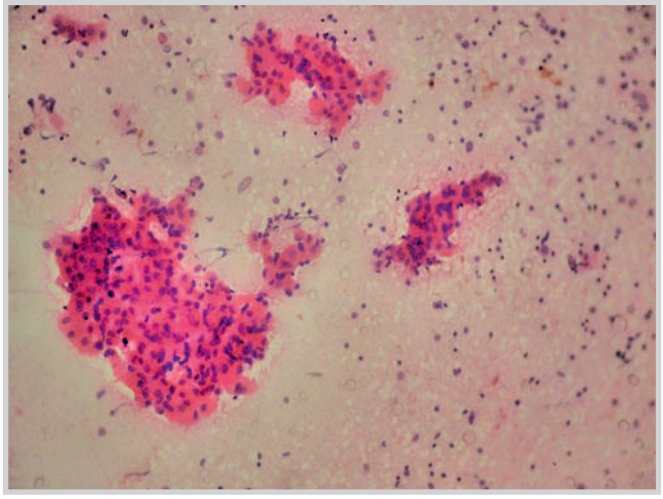

Fig. 25.11: Multiple clusters of epithelial cells along with many discrete myoepithelial cells in pleomorphic adenoma (H & E stain × MP)

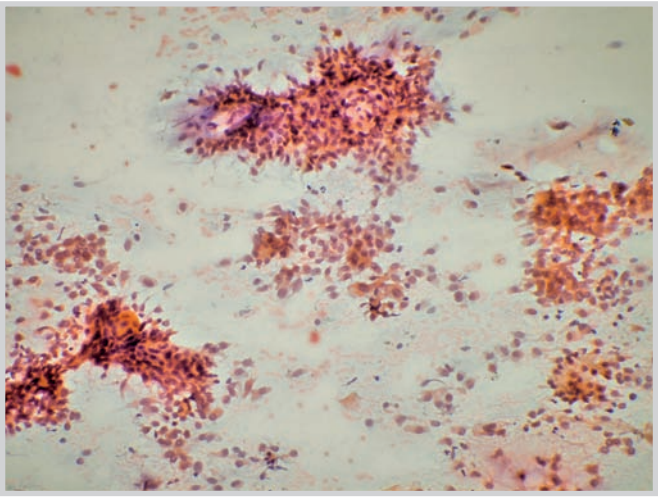

Fig. 25.10: Round to oval cells with moderate amount of cytoplasm in pleomorphic adenoma. The cells are embedded in the stromal material [Papanicolaou's stain × high power (HP)]

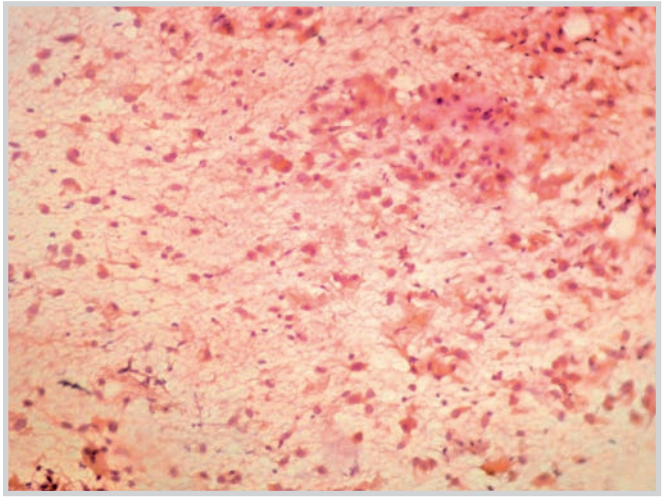

Fig. 25.12: Fine needle aspiration cytology (FNAC) smear of pleomorphic adenoma showing discrete oval to elongated myoepithelial cells with moderate amount of cytoplasm. Nuclei are central to eccentric in position (H & E stain × MP)

may show abundant myxoid stromal material and scanty cellular component. Matrix material in PA is characteristic with certain important features:

- Chondromyxoid and myxoid
- Fibrillar
- Fraying margins
- Cellular: Spindle cells are embedded in the matrix material
- Metachromatic and magenta colored in Romanowsky stain.

Various changes in pleomorphic adenoma:

- Atypical cells: Pleomorphic adenoma may show mild atypical cells in one-fifth of the cases. In absence of necrosis and significant changes in nuclear size, shape and chromatin pattern, these atypical cells should be ignored. However, the presence of necrosis should raise the possibility of carcinoma in PA.
- Metaplastic change: Pleomorphic adenoma may undergo various metaplastic changes such as

BOX 25.9 Pleomorphic adenoma

Cytology

- Pinkish fibrillar chondromyxoid matrix material with frayed indistinct margins.
- Clusters of round, ovoid or plasmacytoid epithelial cells
- Moderate amount of dense cytoplasm.
- Central to eccentric monomorphic nucleus
- Bland chromatin.
- Clusters and discrete spindle shaped myoepithelial cells embedded in mesenchymal stroma.

Immunochemistry: Epithelial cells are positive for CK and myoepithelial cells are positive for CK and vimentin (co-expressed), S-100, GFAP and calponin.

Genetics: PLAG1 gene often shows translocation with CTNNB1 and LIFR.

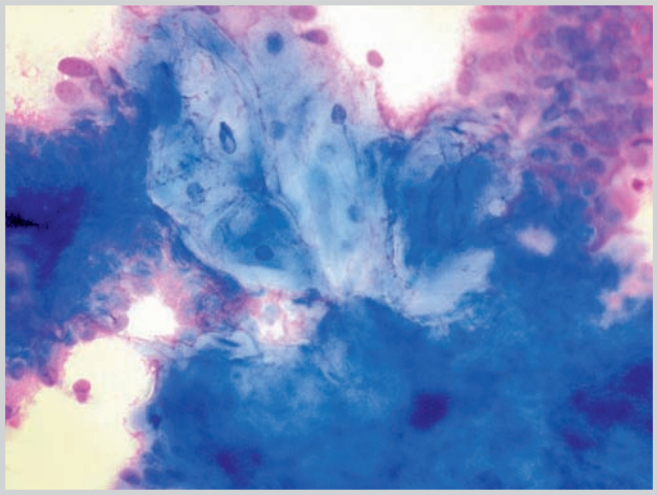

Fig. 25.13: Metaplastic squamous cells in pleomorphic adenoma (MGG stain × MP)

TABLE 25.2: Differentiating features between pleomorphic adenoma and adenoid cystic carcinoma

Cytology	*Type of Tumor*	
	PA	*ACC*
Cell arrangement	Loose cluster and dissociated	Tight cohesive cluster
Cells: Shape	Round and spindle cells	Round
Cytoplasm	Moderate	Scanty
Matrix material: Appearance	Fibrillary pink material.	Amorphous homogenous material.
Cells within the matrix	Matrix contains cells	Acellular Matrix

Abbreviations: PA, pleomorphic adenoma; ACC, adenoid cystic carcinoma

- Squamous metaplasia
- Mucinous metaplasia
- Sebaceous metaplasia
- Oncocytic metaplasia.

Squamous metaplasia is noted about one-fourth cases of PA. The squamous cells are present in small clusters (**Fig. 25.13**). The cells are polyhedral with well-defined cell border. The nuclei of the cells are bland. The keratin pearls may also be seen. At times, PA with extensive metaplastic squamous cells may be mistaken as epidermoid component of MEC. A careful sampling from multiple sites of the tumor is helpful in this condition.

Cystic degeneration: Cystic changes may also be seen in PA. This is due to the degeneration of the tumor mass. The cyst fluid shows cellular debris and histiocytes. Aspiration from multiple sites may avoid any false negative report in such cases.

Differential Diagnosis

- Adenoid cystic carcinoma: The matrix material and the individual cell morphology of the tumor are the most important clues to differentiate ACC from PA. The matrix material is usually in small globules in ACC. The round hyaline globules are surrounded by cells of ACC. These globules are homogenous, well-circumscribed and acellular. The individual cells of ACC show scanty cytoplasm, high nucleocytoplasmic ratio, hyperchromatic nuclei and coarse chromatin, whereas, the cells of PA show moderate cytoplasm and finely granular nuclear chromatin (**Table 25.2**).
- Basal cell adenoma: It will be discussed in BCA section.
- Mucoepidermoid carcinoma: As discussed earlier, the presence of metaplastic squamous cells in PA may simulate low-grade MEC. The squamous cells in PA are usually scanty and show mild nuclear atypia.

Warthin Tumor[22]

Warthin tumor was first described by Aldred Warthin in the year 1929. This tumor is also known as adenolymphoma and cystadenolymphoma and papillary cystadenoma lymphomatosum. It is the second commonest tumor of the salivary gland and it accounts for almost 15% of the salivary gland neoplasm. No monoclonality has been demonstrated in this tumor and probably Warthin's tumor is developmental in origin rather than neoplastic.

Male and female both are almost equally affected by this tumor. The mean age of the tumor is 62 years. The tumors may be bilateral (5–20%). It is almost exclusively seen in the parotid gland. The tumor commonly presents as a soft, painless mass with ill-defined margins. On palpation, the mass shows doughy feel.

Cytology (Figs 25.14 to 25.16)

Aspiration of Warthin tumor yields thick greenish-brown dirty fluid. The smears show reactive lymphocytes and oncocytic cells in the background of amorphous and granular debris. Oncocytic cells are usually present in small cohesive sheets (**Box 25.10**). The individual cells show abundant granular eosinophilic cytoplasm with well-defined cell border. The nuclei are round, monomorphic and eccentrically placed. There may be variable amount of reactive lymphoid cells consisting of mature lymphocytes, follicular center cells and tingible body macrophages. Mast cells are also noted. Nonkeratinizing squamous metaplasia may occur in one-third cases of Warthin's tumor and FNAC smear shows many mature squamous cells along with parakeratotic cells.[23] In addition, the smear may also show inflammatory cells and degenerated cells and may falsely be interpreted as squamous cell carcinoma. Mucinous metaplasia of Warthin tumors shows many mucin secreting cells and is often misinterpreted as MEC.[24]

Differential Diagnosis

- Mucoepidermoid carcinoma: Mucoid material along with metaplastic squamous and mucinous cells in Warthin's tumor may simulate cystic low-grade MEC. Bland nuclear morphology, presence of oncocytes and reactive lymphoid cells favor a diagnosis of Warthin's tumor.
- Squamous cell carcinoma: Metaplastic squamous cells, nuclear debris and dirty background in Warthin's tumor may

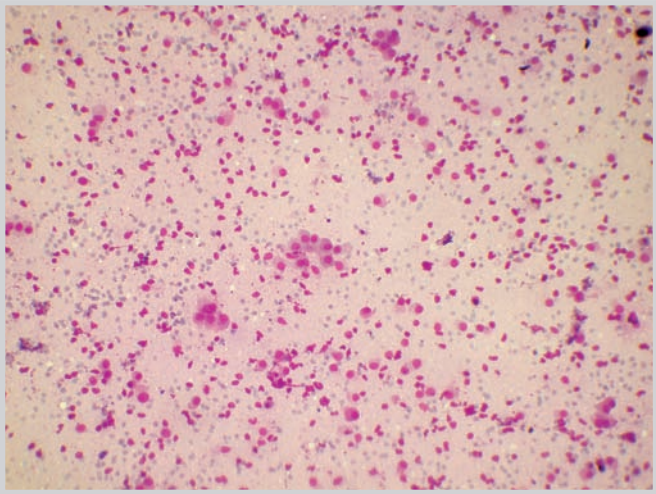

Fig. 25.14A: Warthin tumor: Abundant reactive lymphoid cells and oncocytic cells (MGG stain × LP)

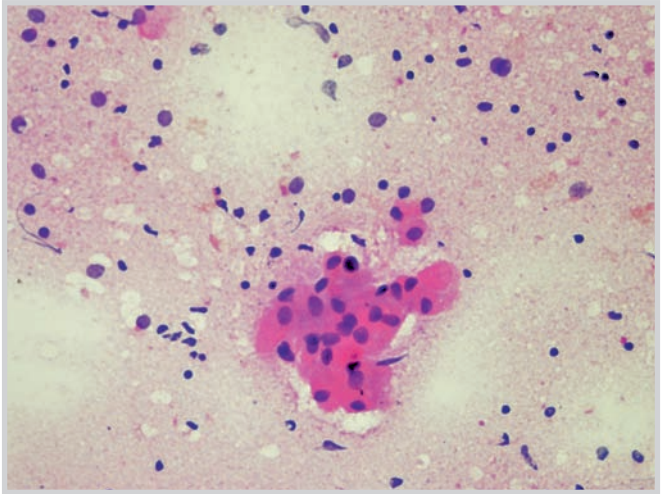

Fig. 25.15: Oncocytic cell in cluster surrounded by reactive lymphoid cells in Warthin tumor. The oncocytes show moderate to abundant cytoplasm and round monomorphic nuclei (H & E stain × MP)

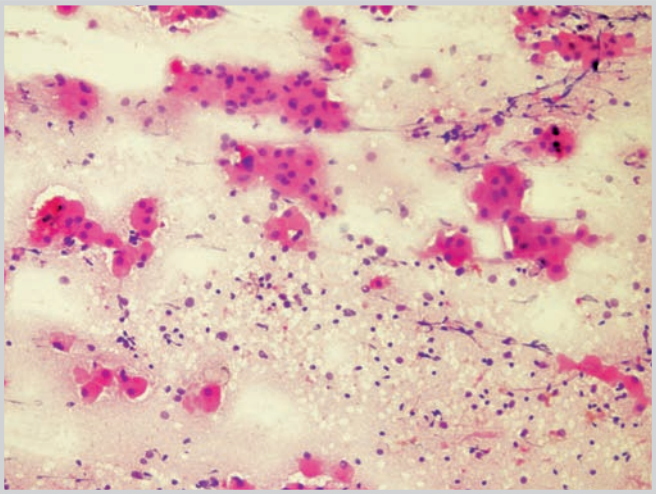

Fig. 25.14B: Warthin tumor: Clusters of oncocytic cells (H & E stain × MP)

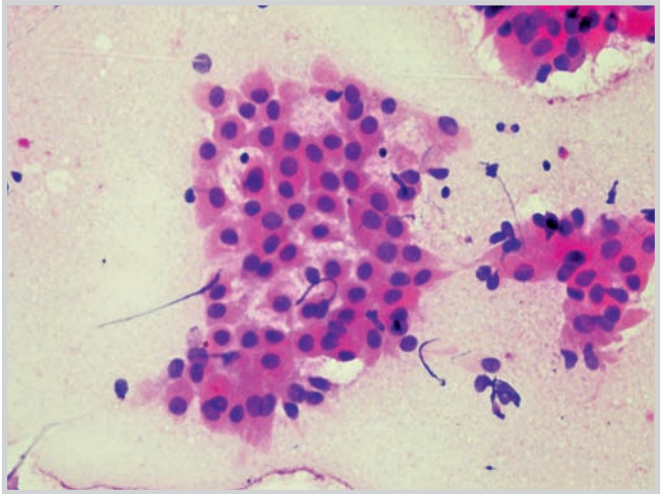

Fig. 25.16: High powered view of the oncocytic cells. The cells have abundant cytoplasm with eccentric nuclei. The nuclei are round and monomorphic (H & E stain × HP)

simulate cystic squamous cell carcinoma. A careful search may reveal oncocytic cells and lymphoid cells in Warthin's tumor.

- Oncocytoma: Abundant oncocytes may pose diagnostic difficulty with oncocytoma.

Basal Cell Adenoma[25]

Basal cell adenomas (BCAs) are rare tumors of the salivary gland. Previously, it was named as monomorphic adenoma. This tumor is characterized by basaloid type of tumor cells and lack of chondromyxoid stroma. BCA accounts for 1–3% of all salivary gland tumors. It predominantly affects older individuals. Majority of the BCAs (more than 75%) occur in the parotid gland and rarely other salivary glands are involved. This tumor usually

BOX 25.10 Warthin tumor

Clinical
- Second common tumors of salivary gland tumors
- Mean age 62 years
- Exclusively within the parotid gland
- One-fifth cases are bilateral
- Doughy feel on palpation
- Probably developmental, not neoplastic

Cytology
- Thin dirty mucoid or greenish-brown fluid
- Many cohesive sheets of oncocytes
- Reactive lymphoid cells
- Squamous and mucinous metaplasia

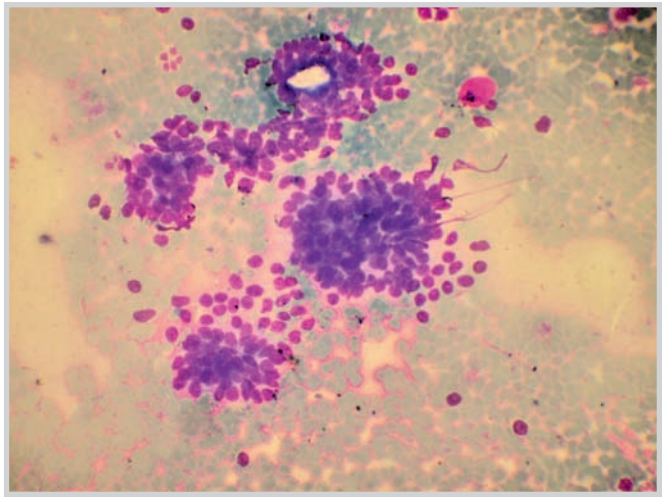

Fig. 25.17: Multiple clusters of basaloid cells with scanty cytoplasm and round monomorphic nuclei in basal cell adenoma (MGG stain × MP)

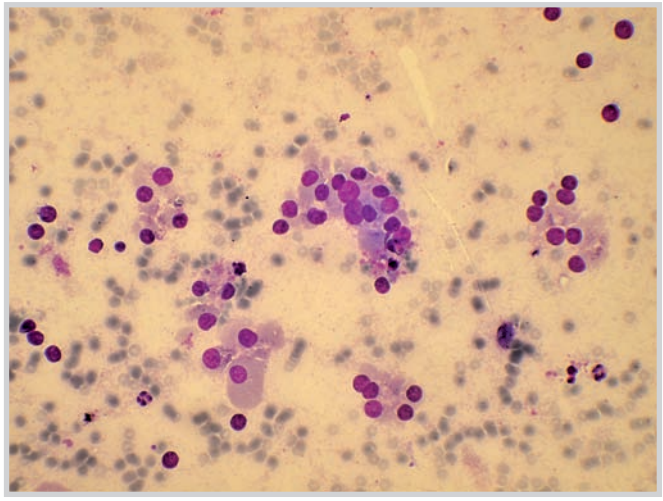

Fig. 25.18: Multiple clusters and discrete oncocytic cells in oncocytoma (MGG stain × MP)

BOX 25.11 Basal cell adenoma

- Cohesive groups of cells
- Peripheral palisading arrangement
- Round nucleus, bland nuclear chromatin and scanty cytoplasm
- Squamous morules
- Scanty homogenous acellular stromal material

presents as solitary, firm, well-defined, mobile nodules. They are usually firm but occasionally cystic.

Cytology (Fig. 25.17)

There are four histological variants of BCA: (1) solid, (2) trabecular, (3) tubular and (4) membranous patterns. Occasionally, there may be admixture of these subtypes. FNAC smear of BCA shows sheets and irregular geographical shaped cords of cells. The cells are often arranged as peripheral palisading manner (**Box 25.11**). There may be sparse metachromatic stained small dense, nonfibrillar intercellular globules of matrix material in between the cells. This matrix material is devoid of any cells. Individual cells in BCA are small, with scanty, pale, basophilic cytoplasm, bland, monomorphic nuclei with granular chromatin and inconspicuous nucleoli. Many naked cells are also seen in the background. In addition, the solid variants of BCA may show squamous morules.

Differential Diagnosis

- Adenoid cystic carcinoma: It is the most important differential diagnosis of BCA and at times it is difficult to differentiate BCA and ACC, particularly in solid type of ACC. Compared to small uniform globules in BCA, the globules are large and varying sized in ACC. These globules are typically surrounded by cells in PA. In addition, the cells show bland nuclear chromatin in BCA compared to hyperchromatic coarse nuclear chromatin in ACC.
- Pleomorphic adenoma: The stromal material is sparse in BCA. In comparison, PA shows abundant chondromyxoid stromal material. Cellular PAs have scanty stromal material and therefore is difficult to differentiate from BCA.

Oncocytoma[25]

Oncocytoma represents 1% of all salivary gland tumors.[27] The peak incidence of the tumor is seventh to ninth decades and the mean age of the tumor is 58 years. This tumor is rarely seen below the age of 50 years. Males and females are equally affected by oncocytoma. More than 80% of the tumor arises from the parotid gland and the rest of the tumor develops from the submandibular gland.[28] The tumor predominantly presents as well-circumscribed painless mobile mass.

This is a benign salivary gland neoplasm that predominantly involves parotid gland (about 75%). The tumor usually presents as a painless mobile mass.

Cytology (Figs 25.18 and 25.19)

Cytology smears show sheets, cords, three-dimensional clusters and dyscohesive abundant oncocytic cells. The individual oncocytic cells are large, polygonal with abundant densely granular eosinophilic cytoplasm and well-defined cell border (**Box 25.12**). On MGG-stained smear, cytoplasm looks deep blue and on Papanicolaou's stain, the cytoplasm appears eosinophilic granular. This dense granular cytoplasm is due to the presence of abundant mitochondria in the oncocytes. Nuclei of the cells are enlarged, monomorphic, round and central in position. Nuclear chromatin is fine with small prominent nucleoli. Unlike Warthin tumor, the background of the smear is clean and free of any lymphocytes. Oncocytoma is difficult to differentiate from the oncocytic hyperplasia on cytology.

Differential Diagnosis

- Warthin tumor: Discussed earlier

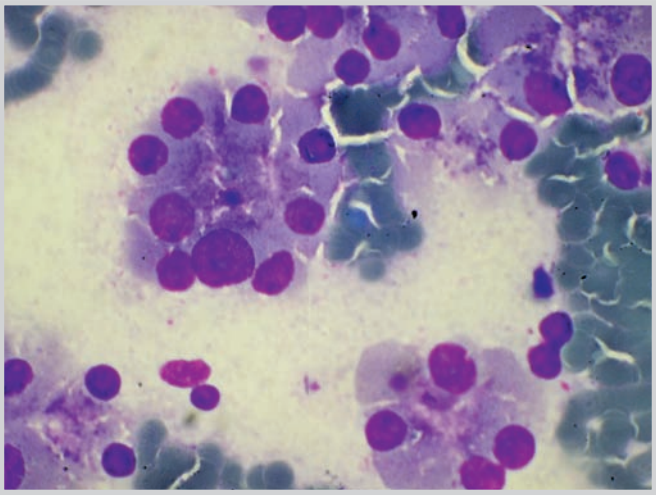

Fig. 25.19: Large, polygonal cells with abundant densely granular eosinophilic cytoplasm and well-defined cell border of oncocytes in oncocytoma (MGG stain × oil immersion)

BOX 25.12 Oncocytoma

- Three-dimensional clusters of oncocytes
- Polygonal cells
- Abundant densely granular eosinophilic cytoplasm
- Monomorphic round nucleus
- Fine chromatin
- Small nucleoli

TABLE 25.3: Cytological features to differentiate oncocytoma and acinic cell carcinoma

Features	*Oncocytoma*	*Acinic cell carcinoma*
Background	Clean	Naked nuclei
Cytoplasm	Waxy, granular	Pale
Cytoplasmic vacuoles	Absent	Present
PAS	Negative	Positive
PTAH	Positive	Negative

Abbreviations: PAS, periodic acid-schiff; PTAH, phosphotungstic acid-hematoxylin

- Acinic cell carcinoma: Acinic cells in acinic cell carcinoma may be mistaken as oncocytes. The cytoplasm of the acinic cell is pale, delicate and often vacuolated. The vacuoles contain zymogen granules and are positive for periodic acid-schiff (PAS) stain. Whereas, the oncocytes have waxy, granular cytoplasm. The cytoplasm is rich in mitochondria and is positive for phosphotungstic acid-hematoxylin (PTAH) stain (**Table 25.3**).
- Tumors with oncocytic changes: Oncocytic metaplasia is noted in PA, MEC and Warthin's tumor. However, they usually do not pose significant problem as these tumors also contain other components.

Myoepithelial Tumors[29-33]

Myoepithelial tumor is a rare benign neoplasm and comprises only 1.5 % of all salivary gland neoplasms. Both male and female are equally affected by myoepithelioma.[32] The age range of the patient is from first decade to ninth decade and the mean age of the patient is 44 years. Myoepithelioma may occur both in major and minor salivary gland. However, parotid and minor salivary glands of palate are the commonest sites of involvement. The patients usually present with a slow growing painless mass.

Histological types of myoepithelioma are spindle, plasmacytoid or hyaline, epithelioid and clear cell.[33] Occasionally, there may be combination of more than one type.

Cytology (Box 25.13)

Cytology smear of myoepithelioma depends on the variant of myoepithelioma. In spindle cell type of the tumor, the FNAC smears show abundant cohesive groups and isolated spindle cells. The smear shows striking absence of chondromyxoid material. Individual cells are elongated with ovoid to fusiform nuclei. The nuclear ends are more rounded than pointed. The chromatin is fine and nucleoli are indistinct. Occasional cells show nuclear groove. Cytoplasm of the cell is pale and scanty.

The smears of plasmacytoid variant (**Figs 25.20** and **25.21**) show dissociated plasmacytoid cells with abundant cytoplasm and eccentric nucleus. These cells are much larger than the plasma cells and they have fine chromatin. The nuclei lack typical cartwheel appearance and perinuclear hof of plasma cells.[34,35]

BOX 25.13 Myoepithelial tumors

Cytology
- Rich in cells
- Lack of chondromyxoid stroma
- Spindle cell type
- Cohesive clusters and dissociated spindle cells
- Elongated nuclei, fine nuclear chromatin and inconspicuous nucleoli
- Nuclear grooves
- Scanty pale cytoplasm
- Plasmacytoid (hyaline) myoepithelial cells
- Dissociated round to oval cells.
- Plasmacytoid cells with abundant cytoplasm and eccentric nucleus
- Fine nuclear chromatin, no perinuclear hof

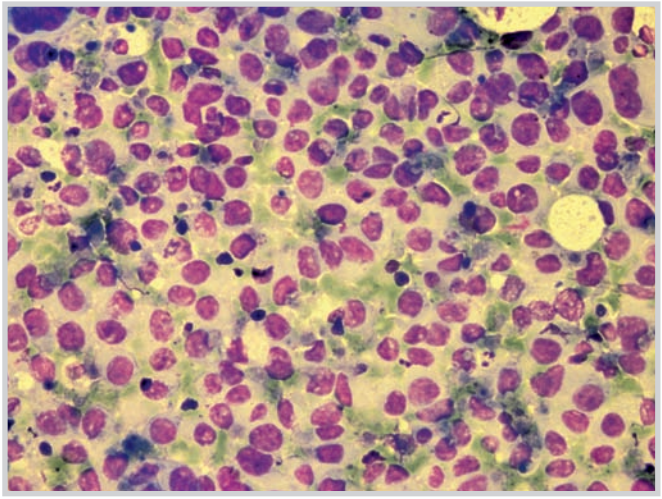

Fig. 25.20: Plasmacytoid variant of myoepithelial tumor showing dissociated plasmacytoid cells with abundant cytoplasm and eccentric nucleus (MGG stain × MP)

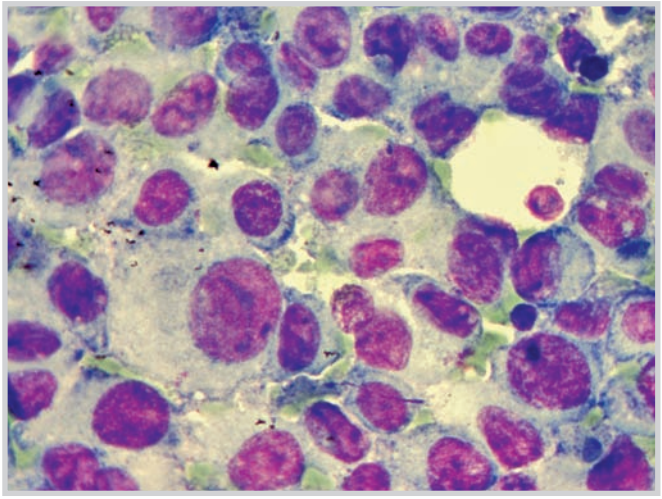

Fig. 25.21: Individual cell morphology of myoepithelial cells is better viewed in higher magnification in myoepithelial tumor (H & E stain × HP)

Immunocytochemistry

The tumor cells are positive for cytokeratin (CK) 7 and 14. The spindle cells in myoepithelioma are also positive for alpha smooth muscle actin, calponin, muscle specific actin, glial fibrillary acidic protein (GFAP) and S-100. In addition, myoepithelial cells are also positive for p63.

Differential Diagnosis

- Schwannoma: Spindle cell variants of myoepithelioma closely mimic schwannoma. Nuclei of schwannoma are elongated with wavy pointed ends rather than round ends in cells of myoepithelioma. At times, rows of palisaded nuclei in hypocellular fibrillar stroma, called as Verocay bodies, may also be seen. In case of difficulty, a panel of immunocytochemistry particularly myoepithelial markers may be helpful. **Table 25.4** shows differential diagnosis of spindle cell lesions of the salivary gland.
- Plasma cell tumor: Plasma cell tumor is the close differential diagnosis of plasmacytoid type of myoepithelioma. However, the myoepithelioma cells show lack of nuclear characters of plasma cells such as cart wheel chromatin and perinuclear hof.
- Pleomorphic adenoma with predominantly spindle cells (**Fig. 25.22**): Pleomorphic adenoma with scanty epithelial cell may pose diagnostic difficulty.[36] This may happen due to improper sampling from the predominant myoepithelial rich area of PA. The stromal cells of PA are almost similar in morphology as that of myoepithelioma and in absence of chondromyxoid material it is very difficult to distinguish PA with spindle cell rich aspirate from myoepithelioma.

Malignant Tumor

Adenoid Cystic Carcinoma[37-40]

Adenoid cystic carcinoma is a slow growing tumor and represents 5–10% of all salivary gland neoplasms. It comprises about 10–20% of salivary gland malignancies. ACC commonly affects parotid, submandibular gland and minor salivary glands in the palate. ACC may also occur in nasal cavity and sinuses, tongue, buccal mucosa and lip. Uncommonly, ACC may also affect other sites such as skin,

TABLE 25.4: Differential diagnosis of spindle cell lesions in salivary glands

Features	*Myoepithelioma (spindle cell type)*	*Pleomorphic adenoma*	*Schwannoma*
Verocay bodies	Absent	Absent	Present
Chondromyxoid stroma	Scanty to absent	Abundant	Absent
Nucleus	Elongated with rounded ends	Elongated with rounded ends	Wavy and pointed
Immunostaining: S-100 Keratin Calponin Smooth muscle actin	 Weak Positive Positive Positive	 Weak Positive Positive Positive	 Strong Negative Negative Negative

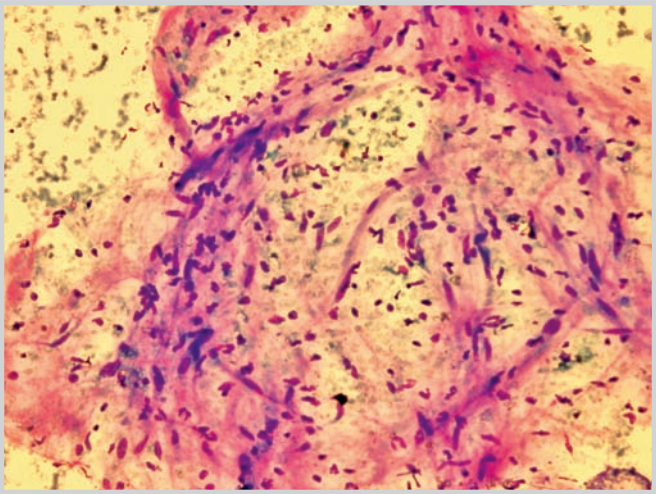

Fig. 25.22: Spindle cell type of pleomorphic adenoma showing elongated spindle cells in pinkish connective tissue stroma (MGG stain × HP)

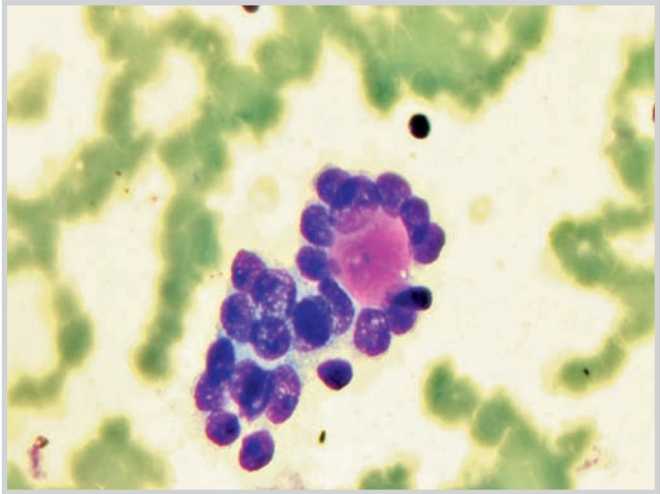

Fig. 25.24: Pinkish homogenous globules surrounded by round mildly pleomorphic cells in cribriform variant of adenoid cystic carcinoma (MGG stain × MP)

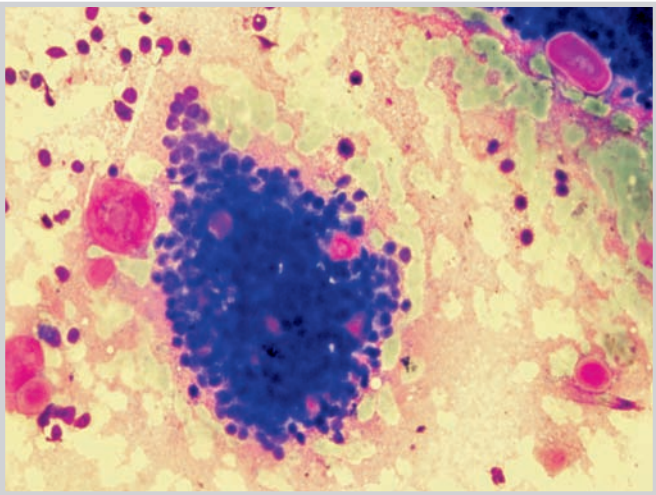

Fig. 25.23: Cribriform appearance of adenoid cystic carcinoma on cytology smear (MGG stain × MP)

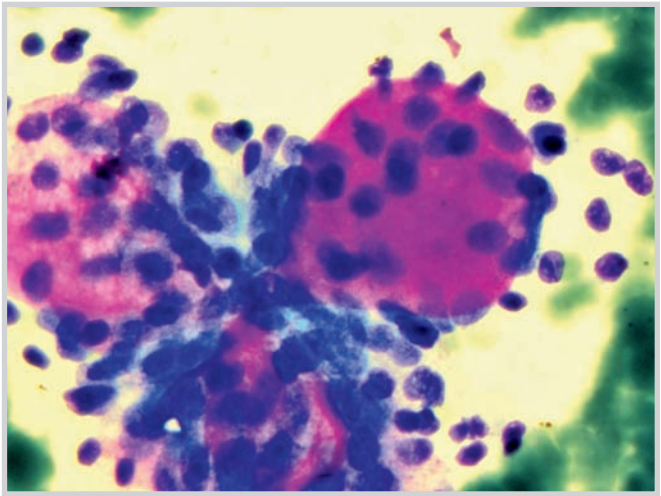

Fig. 25.25: Adenoid cystic carcinoma: Higher magnification of pinkish globule surrounded by cells (MGG stain × HP)

lung, prostate, breast, and the lower part of the female genital tract. Females are slightly more affected than male. The peak incidence of the tumor is in the fourth to six decades of life. The patient commonly presents as slow growing swelling followed by pain in the ear as the tumor often infiltrates local nerves.

Cytology (Figs 25.23 to 25.26)

There are three histologic subtypes of ACC: (1) cribriform, (2) tubular and (3) solid. The solid form is considered as high grade type of ACC because it often follows an aggressive course. However, the solid variety of ACC is more difficult to diagnose in FNAC than the other two subtypes (**Box 25.14**). The cribriform and tubular subtypes of ACC show characteristic matrix material. On MGG smear the matrix material looks deep, homogenous, magenta color globules, or branching tubules with sharp outline and devoid of any cells (**Box 25.15**). On Papanicolaou's stain, this matrix material appears as colorless area. The globular material is surrounded by basaloid cells. The individual basaloid cells are round with scanty clear cytoplasm. The nuclei are monomorphic, hyperchromatic with angulated margin.

In histological solid variant of ACC, the characteristic metachromatic matrix material is scanty to absent. The cytology smears show three-dimensional clusters of basaloid cells with scanty clear cytoplasm and dark angulated nuclei. Nuclei are hyperchromatic and show mild to moderate pleomorphism. In addition, variable degree of mitotic activities and apoptosis may also be noted.

Differential Diagnosis

- Pleomorphic adenoma
- Basal cell adenoma
- Polymorphous low-grade adenocarcinoma.

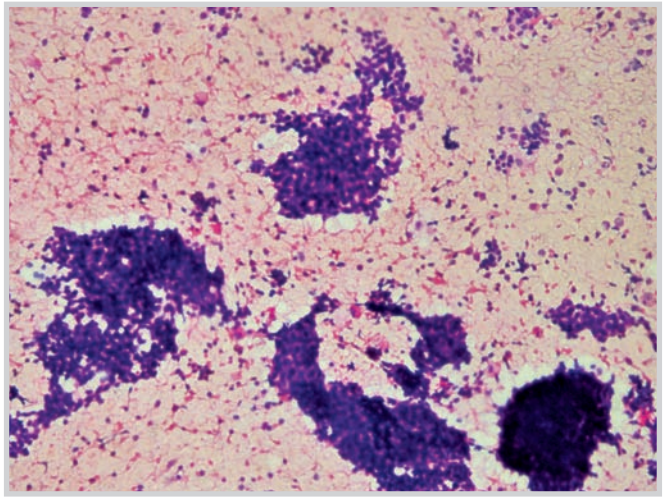

Fig. 25.26: Clusters and dissociated round cells in solid variant of adenoid cystic carcinoma (H & E stain × MP)

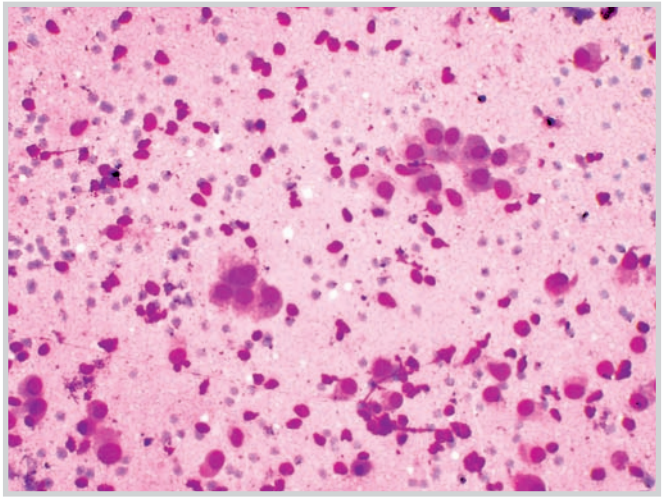

Fig. 25.27: Small aggregates and discrete acinar cells in acinic cell carcinoma. Background of the smear shows many naked nuclei (MGG stain × MP)

BOX 25.14 **Adenoid cystic carcinoma**

- Multiple variables sized globular, spherical or tubular homogenous, acellular magenta colored matrix material
- Globules are surrounded by cells
- Clusters and dissociated small cells
- Scanty cytoplasm
- Round monomorphic nuclei
- Hyperchromatic
- Coarse chromatin

Immunocytochemistry: Ki67: High Ki67 index,
c-Kit over expression.

BOX 25.15 **Important features of matrix in adenoid cystic carcinoma (ACC)**

- Homogenous
- Globular or branching tubular
- Sharp margin
- Acellular: Inside matrix—no cells
- Surrounded by basaloid cells

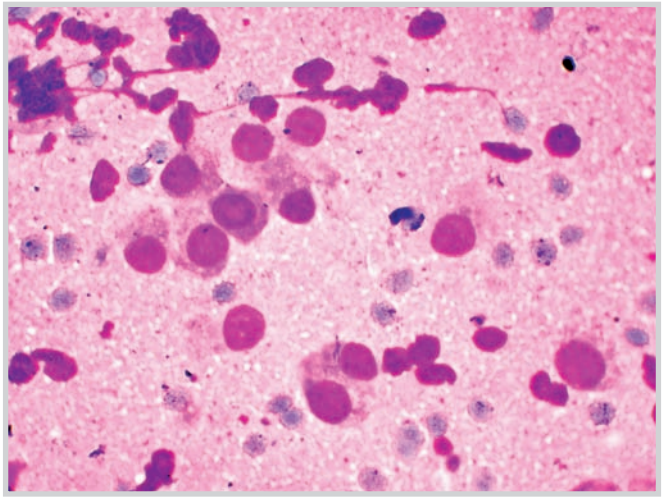

Fig. 25.28: Higher magnification of the acinic cell carcinoma showing abundant eosinophilic cytoplasm and round central to eccentric nuclei (MGG stain × oil immersion)

Acinic Cell Carcinoma[41,42]

Acinic cell carcinoma is the malignant neoplasm of the epithelial cell with serous acinar cell differentiation and constitutes only 1–3% of the malignant salivary gland tumors. Females are slightly more affected by acinic cell carcinoma than male and no age group is exempted by this tumor. The peak incidence of the tumor is in third decades of life. About 80–90% of tumor arises in the parotid gland and a small group of tumor develops from the minor salivary glands. The patient classically presents as a single, mobile and painless mass. The duration of the symptoms is usually few months. However, some patient may give the history of swelling for 10 years or more. About one-third of the cases may have vague pain and about 10% patients may also show facial paralysis at the time of presentation.[43,44]

Cytology (Figs 25.27 and 25.28)

Several histopathological subtypes of acinic cell carcinoma are recognized:

- Solid or lobular
- Microcystic
- Papillary-cystic
- Follicular growth patterns.

The tumor also contains various types of cells such as:

- Serous acinar

BOX 25.16 Acinic cell carcinoma

- Abundant cohesive clusters of acinar cells
- Acinar like arrangement
- Large polygonal cells
- Abundant finely vacuolated cytoplasm
- Naked tumor nuclei
- Occasionally lymphocytes and psammoma bodies
- Clean background

- Nonspecific glandular
- Intercalated ductal
- Vacuolated
- Clear.

Therefore, the FNAC smears may show variable features depending on the primary architectural and cell type of the tumor. However, all the tumors show predominant acinar cell differentiation.

Fine needle aspiration cytology smear is usually rich in cells. The cells are arranged in three-dimensional clusters and sheets in a clean background (**Box 25.16**). Occasionally, follicular or micropapillary structures may be noted. The cells may also be arranged around thin capillaries. The individual cells are polygonal with abundant finely vacuolated cytoplasm. Nuclei are rounded, monomorphic, centrally placed with a small distinct nucleoli. The cytoplasmic vacuole is subtle and better seen in MGG stain. The vacuoles contain PAS positive diastase-resistant zymogen granules. The cytoplasmic vacuole is one of the important findings and should not be overlooked. Careful observations help to differentiate the acinic cell from oncocytes (**Table 25.3**). The acinic cells are delicate so they may breakdown and the background often shows many bare naked nuclei. About 10% cases of acinic cell carcinoma may also background lymphocytes.

In case of papillary cystic variety of acinic cell carcinoma, the aspirate may yield fluid and smears of which shows foamy vacuolated hemosiderin laden macrophages and granular debris (**Box 25.17**). A complex architecture of branching papillary structure is also noted in such cases. Papillary cystic variant of acinic cell carcinoma may also show psammoma bodies. These finding may mislead to the false diagnosis of MEC or Warthin's tumor.[43,45]

Differential Diagnosis

- Benign salivary gland aspirate: The presence of benign looking acinar cells in acinic cell carcinoma may give the false impression of benign salivary gland aspirate and vice versa. However, the normal acinar cells are polarized in lobular groups unlike three-dimensional clusters of cells in acinic cell carcinoma.
- Oncocytic tumor: Described in oncocytic tumor.
- Mucoepidermoid carcinoma: Papillary cystic variant of acinic cell carcinoma may be mistaken as MEC. The presence of malignant squamous cell is the helpful diagnostic feature of MEC.
- Warthin tumor: As discussed earlier, occasional lymphoid cells in the background of acinic cells may simulate Warthin tumor. Oncocytes in Warthin tumor show abundant granular cytoplasm and no vacuoles are demonstrated within the cytoplasm. If necessary PAS positive-diastase resistant, material can be demonstrated in the cell block preparation.
- Oncocyoma: Abundant acinic cells may simulate oncocytes and may mislead the diagnosis of oncocyoma.
- The differential diagnoses of acinic cell carcinoma are highlighted in **Table 25.5.**

BOX 25.17 Cytological features of papillary cystic adenoid cystic carcinoma (ACC)

- Cystic with cellular debris
- Paucicellular
- Small papillary structures and flat sheets
- Vacuolated histiocyte like cells
- Monotonous polygonal neoplastic cells with enlarged nuclei
- Rare oncocytic cells:

D/D: Mucoepidermoid carcinoma, PA, Warthin's tumor, retention cyst and metastatic renal cell carcinoma

Abbreviations: D/D, differential diagnosis; PA, pleomorphic adenoma

TABLE 25.5: Oncocytes versus acinic cells of acinic cell carcinoma

Features	*Acinic cell*	*Oncocytes*
Cell margin	Indistinct	Well-defined
Cytoplasm character	Fine vacuolations	Eosinophilic granular
Cytoplasm quantity	Abundant	Moderate
PAS with diastase	Positive	Negative
PTAH	Negative	Positive
Electron microscopy	Zymogen granules	Mitochondria

Abbreviations: PAS, periodic acid-schiff; PTAH, phosphotungstic acid-hematoxylin

Mucoepidermoid Carcinoma[46]

Mucoepidermoid carcinoma is the commonest malignant tumors of the salivary gland and represents 10%–15% of salivary gland tumors.[47] MEC occurs in age groups from children to old, and mean age of the patient is 45 years. There is mild female predilection of this tumor. About 50% of MEC arises from the major salivary gland and rest of the tumor arises from buccal mucosa and palate.[48,49]

The patients usually present with painless, firm, fixed, mass.

Cytology (Figs 25.29 to 25.31) (Box 25.18)

Mucoepidermoid carcinoma is divided on histology as low, intermediate and high-grade tumor. The high-grade MEC is aggressive compared to low-grade MEC.[50,51] The cytological features of different grades of MEC show considerable

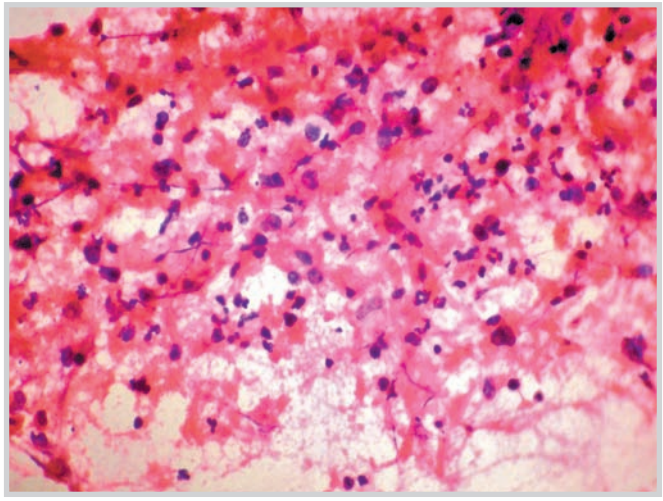

Fig. 25.29: Dirty background in mucoepidermoid carcinoma. Smear shows many tumor cells and polymorphs (H & E stain × MP)

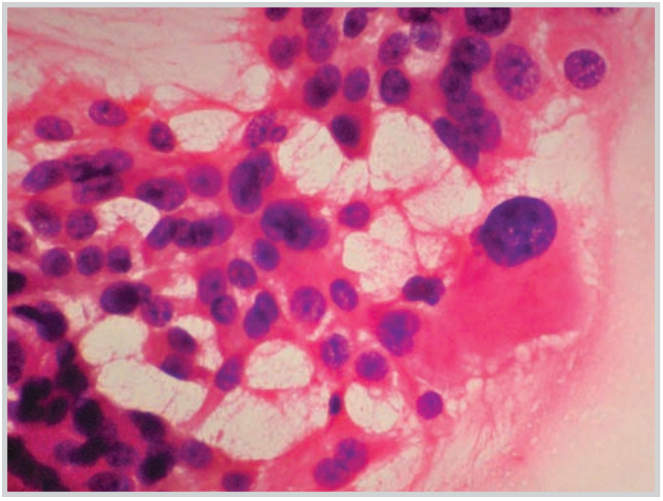

Fig. 25.31: Epidermoid cells with enlarged hyperchromatic nuclei in mucoepidermoid carcinoma (H & E stain × HP)

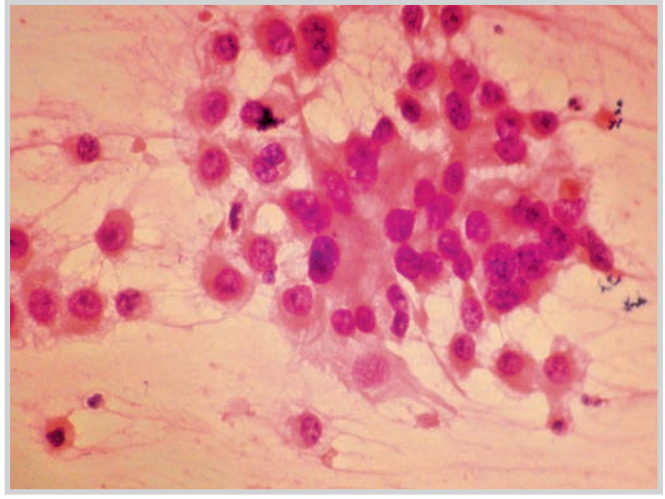

Fig. 25.30: Malignant cells with vacuolated cytoplasm along with intermediate type of squamoid cells in mucoepidermoid carcinoma (H & E stain × HP)

differences. Low-grade MEC is usually cystic and often gives rise to false negative diagnosis.

Fine needle aspiration cytology smears show predominantly three types of cells: (1) squamoid cells, (2) intermediate cells and (3) mucus secreting cells. The back ground of the tumor is dirty with mucus and debris.

The squamoid or epidermoid cells are polygonal in shape with orangeophilic cytoplasm in Papanicolaou's stain. The intermediate cells are smaller in size, columnar to polygonal and have enlarged hyperchromatic nuclei with high nucleocytoplasmic ratio. The mucus secreting cells are round with abundant vacuolated cytoplasm. The nuclei are eccentrically placed and indented and often give signet ring like appearance. The nuclei of mucus secreting cells of MEC show low nucleus-to-cytoplasm (N/C) ratio and almost no nuclear enlargement. These cells are easily mistaken as histiocytes. However, the histiocytes show multiple vacuoles, phagocytosed material and often centrally placed nuclei. In addition, there may be clear cell or oncocytic cells. Oncocytic variant of MEC

BOX 25.18 Mucoepidermoid carcinoma

- Clusters and dissociated cells with vacuolated cytoplasm
- Intermediate squamous cells
- Gradual transition of squamoid to mucus producing cells
- Dirty background produced by debris and mucus
- Histiocytes and background lymphocytes
- Mucus secreting cells are positive for mucicarmine or Alcian blue

shows relatively large amount of oncocytes admixed with mucus secreting cells in a clean mucoid background. The cytology smear may be misinterpreted as oncocytoma. However, the presence of mucus secreting cells is the key feature of correct diagnosis.

In case of low-grade MEC, considerable cystic changes are seen and FNAC may yield fluid. The smears show mucus secreting cells and many histiocytes in a background of mucus. About one-fifth of MEC shows background lymphocytes. Malignant squamous and intermediate cells are usually less in low-grade tumor and these cells show mild nuclear enlargement and pleomorphism. The low-grade MEC is the common source of under diagnosis.[52]

The low-grade MEC may often be mistaken as retention cyst, Warthin tumor and cystic PA. In high-grade MEC, the malignant epidermoid cells are more in amount and these cells show moderate to marked nuclear enlargement and pleomorphism. The high-grade MEC is easily recognizable malignant tumor in the salivary gland.

Differential Diagnosis

- Acinic cell carcinoma: Discussed earlier
- Warthin tumor: Discussed earlier
- Retention cyst: Mucus and cellular debris along with inflammatory cells may cause diagnostic confusion with retention cysts of the salivary gland.
- Metastatic squamous cell carcinoma with cystic changes: Metastatic squamous cell carcinoma in an intraparotid lymph node may often simulate MEC. A careful history may avoid this mistake.

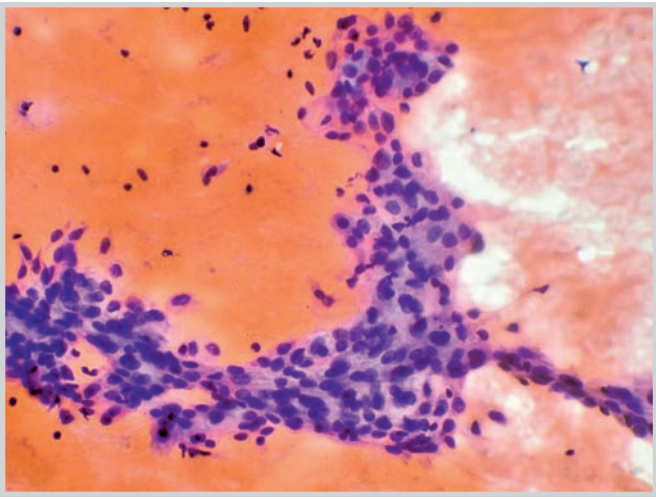

Fig. 25.32: Long papillary like cluster in polymorphous low-grade adenocarcinoma (MGG stain × MP)

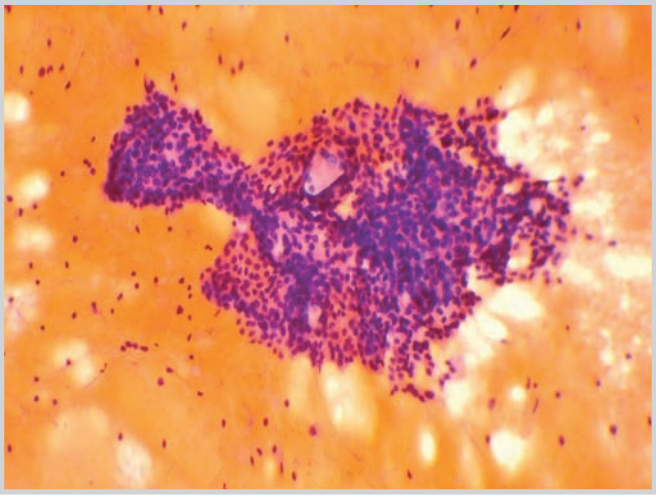

Fig. 25.33: Cluster of cells with round relatively monomorphic nuclei and moderate amount of cytoplasm in polymorphous low-grade adenocarcinoma (MGG stain × MP)

Polymorphous Low-grade Adenocarcinoma[47,53,54]

Polymorphous low-grade adenocarcinoma represents about 26% of all carcinomas of the salivary gland.[53] Females are more commonly affected than male. The age range of the patient varies from second decade to ninth decade. However, majority of the patients are in between 50 years and 70 years of age. Mean age of the patient is 59 years. PLGA commonly affects the minor salivary glands and 60% of the tumor develops in palate.[54] It is a less aggressive malignant tumor of the salivary gland with better prognosis. The patient usually presents as painless mass in the palate.

Cytology[55] (Figs 25.32 and 25.33)

Fine needle aspiration cytology smears of PLGA show clusters, papillary, trabecular and discrete arrangement of cells. The cells of PLGA are cuboidal to columnar rather than basaloid cells of ACC. The individual cells are monomorphic cuboidal to columnar with moderate amount of eosinophilic pale cytoplasm (**Box 25.19**). The nuclei of the cells are relatively monomorphic having fine stippled nuclear chromatin and inconspicuous nucleoli. The extracellular matrix material of PLGA is scanty and is fibrillar in appearance similar to that of PA. However, spherical acellular homogenous globules like stromal material similar to ACC are also seen in PLGA. There are overlapping cytomorphological features of PA, ACC and PLGA. Therefore, it is very difficult to diagnose PLGA in FNAC. However, the presence of papillary and trabecular pattern of cells are helpful features to diagnose PLGA in FNAC.

BOX 25.19 **Polymorphous low grade adenocarcinoma**

Cell arrangement:
- Papillary
- Ductal
- Trabecular
- Discrete

Morphology:
- Scanty to moderate pale cytoplasm
- Round to oval nucleus
- Fine stippled nuclear chromatin
- Inconspicuous nucleoli
- Scanty matrix material

D/D: PA, ACC

Abbreviations: D/D, differential diagnosis; PA, pleomorphic adenoma; ACC, adenoid cystic carcinoma

Differential Diagnosis

Adenoid cystic carcinoma: The presence of hyaline stromal globules and the small monomorphic cells may simulate ACC. However, in contrast to ACC that shows basaloid cells, the cells of PLGA are more cuboidal to columnar and contains relatively more eosinophilic cytoplasm.

Epithelial-Myoepithelial Carcinoma

This malignant tumor is composed of both ductal and myoepithelial malignant cells and it gives a biphasic pattern. Epithelial-myoepithelial carcinoma is an uncommon tumor and represents only 1% of all salivary gland tumors. It occurs in a wide range of age. However, the peak incidence of the tumor is six to seventh decades. Epithelial-myoepithelial carcinoma occurs twice in females than in males. Majority of the epithelial-myoepithelial carcinomas (60–80%) arise in the parotid gland and rest of the tumors arise in the minor salivary glands of the oral cavity. The patient usually presents as painless slowly growing mass in the parotid gland for months to even years.

Cytology[56-59]

Cytology of the epithelial-myoepithelial carcinoma usually gives a bland outlook. However, a possibility of malignancy is considered in most of the studies.[56-59] The cytology smears usually

BOX 25.20 Epithelial-myoepithelial carcinoma

- Biphasic pattern
- Myoepithelial cells:
  - Loose cohesive cluster
  - Large polygonal clear cell
  - Round, monomorphic nuclei
  - Mild nuclear enlargement
  - Fine chromatin
- Epithelial cells:
  - Scanty
  - Small cuboidal
  - High N/C ratio
- Stromal material:
  - Spherical hyaline globules
  - Acellular

D/D: PA, ACC

Abbreviations: D/D, differential diagnosis; PA, pleomorphic adenoma; ACC, adenoid cystic carcinoma

show a biphasic pattern consisting of small ductal epithelial and myoepithelial cells (**Box 25.20**). The myoepithelial cells are present in loose cohesive sheets and three-dimensional spherical clusters. The individual cells are large polygonal with abundant clear vacuolated cytoplasm. The nuclei are monomorphic, rounded and enlarged. Nuclear chromatin is finely dispersed with distinct nucleoli.

The other population of cells is ductal. These cells form cohesive sheets or tubular structures. The ductal cells are small, cuboidal with well-defined border. The nuclei are round hyperchromatic with high N/C ratio. The nuclear chromatin is granular with prominent nucleoli. In some cases, the biphasic pattern may be lost and the smears show apparently one population of cells. This may be due to the breakdown of the cytoplasm of the cell and dispersed-naked nuclei on the background of the smear.[58] In addition, the FNAC smear of the tumor also shows background stromal material. The hyaline globules may be tightened and spherical and surrounded by the small cuboidal cells. At times, these may closely mimic ACC.[59]

Carcinoma Ex-pleomorphic Adenoma

Carcinoma ex-pleomorphic adenoma (Ca Ex-PA) is a malignant epithelial tumor that arises from previously existing PA. It is also known as malignant mixed tumor. Ca Ex-PA represents 12% of malignant salivary gland tumors. The tumor commonly occurs in sixth and seventh decades of life.[60] Ca Ex-PA commonly involves the parotid gland. The patient usually presents with history of a sudden rapid enlargement of a longstanding swelling of the parotid gland.

Cytology

Cytology smear is usually cellular. The smear shows a high-grade carcinoma usually adenocarcinoma of ductal type. The background shows metachromatic fibrillar matrix material. Myoepithelial cells are embedded in this material (**Box 25.21**). The malignant nature of the tumor is easy to identify. However, for the diagnosis of Ca Ex-PA, a pre-existing diagnosis of PA is required. On cytology alone, it is difficult to distinguish the tumor from high-grade MEC and metastatic adenocarcinoma.[61]

BOX 25.21 Carcinoma ex-pleomorphic adenoma

- History of pre-existing PA
- High-grade carcinoma of ductal type
- Metachromatic fibrillar matrix with myoepithelial cells

D/D: High-grade MEC, metastatic carcinoma

Abbreviations: D/D, differential diagnosis; PA, pleomorphic adenoma; MEC, mucoepidermoid carcinoma

Myoepithelial Carcinoma

Myoepithelial carcinoma is the rare malignant counterpart of myoepithelioma. This represents less than 2% of all salivary gland carcinoma. Both males and females are equally affected by myoepithelial carcinoma. About 75% myoepithelial carcinoma arises in the parotid gland.[62]

Cytology

The presence of pleomorphism, coarse chromatin, mitosis and necrosis in a myoepithelioma suggest myoepithelial carcinoma.[29]

Salivary Duct Carcinoma

This is an uncommon malignancy of the salivary gland and comprises 9% of salivary malignancies. The males are four times more affected than females.[63] The parotid gland is commonly involved by this tumor and the patient presents with a rapidly growing mass. This is an aggressive adenocarcinoma.

Cytology

Fine needle aspiration cytology smears show sheets and papillary group of cells. The individual cells are large, polygonal with abundant vacuolated cytoplasm. The nuclei of the cells show marked pleomorphism, hyperchromasia and prominent nucleoli. In addition, there may be atypical mitosis, increased mitotic activity and necrosis. The malignant nature of the tumor is easily recognizable. However, the tumor simulates any other high-grade malignant tumor.[64,65]

Adenocarcinoma not Otherwise Specified

This tumor represents 17% of all salivary gland malignancies and predominantly involves the parotid gland. The tumor cells exhibits ductal differentiation.[10] FNAC smears show recognizable malignant cells with features of adenocarcinoma but there is lack of definitive evidence of other specific subtypes.

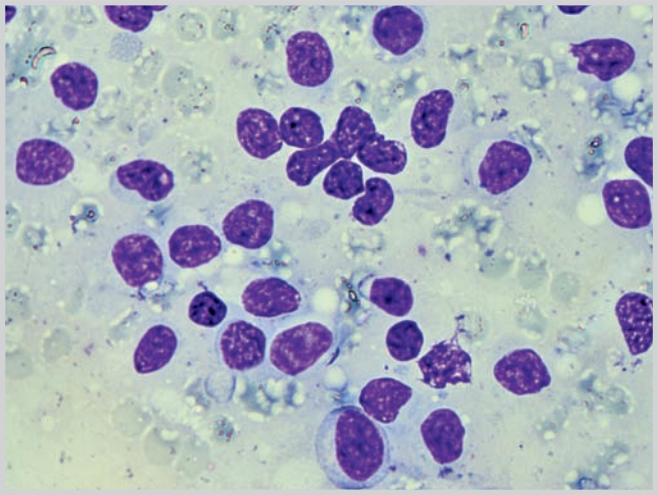

Fig. 25.34: Mucosa-associated lymphoid tissue lymphoma (MALTOMA) in salivary gland. The lymphoid cells are round with scanty cytoplasm and mildly pleomorphic nuclei with condensed chromatin (MGG stain × oil immersion)

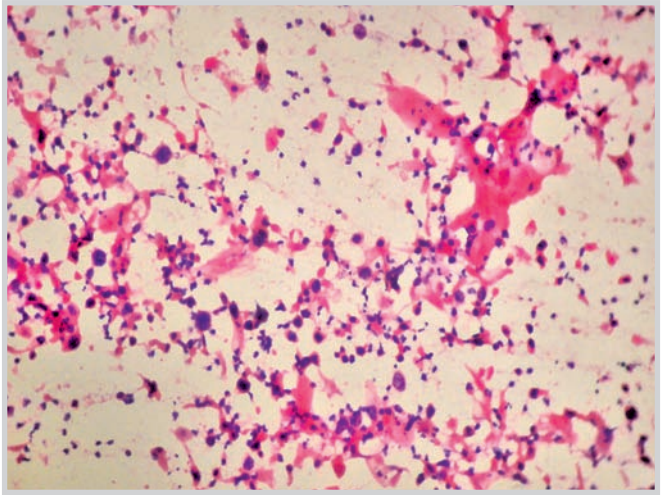

Fig. 25.35: Fine needle aspiration cytology (FNAC) smear of metastatic squamous cell carcinoma of parotid gland (H & E stain × HP)

Lymphoma of the Salivary Gland

Primary NHL of the salivary gland is uncommon and represents only 5% of all extranodal lymphoma.[66] For the definition of primary NHL of the salivary gland, two essential features are required:

- The main bulk of the lymphoma should be in the salivary gland.
- The parenchyma of the salivary gland should be involved.

The most of NHL are B cell type and the common subtypes are:[16]

- Extranodal marginal zone lymphoma of the mucosa-associated lymphoid tissue (MALT) type
- Follicular lymphoma
- Diffuse large B cell lymphoma.

All the B cell, NHL cases are positive for CD45 and CD20. In addition, the lymphoid cells show light chain restriction.

Extranodal marginal zone lymphoma of the MALT type is a low-grade NHL. Cytology smears show discrete monomorphic small to intermediate sized lymphoid cells (**Fig. 25.34**). The cytoplasm of the cells is scanty. Nuclei are small round, hyperchromatic with indented nuclear margin and indistinct nucleoli.

Diffuse large B cell lymphoma shows discrete large immature lymphoid cells. The nuclei show fine nuclear chromatin with prominent nucleoli.

Differential Diagnosis

The common differential diagnoses of MALT NHL of the salivary gland are:

- Lymphoepithelial sialadenitis
- Chronic sialadenitis
- Intraparotid reactive lymph node.

Fine needle aspiration cytology smear of MALT NHL shows monomorphic population of lymphoid cells in contrast to lymphoid cells in variable stage of maturation in LESA. The demonstration of the light chain restriction may be necessary to confirm NHL in certain cases. In all cases of NHL, a detailed immunocytochemistry in cell block or FCM from the aspirate is required for further subtyping.

METASTATIC CARCINOMA

Various other malignancies may metastasize in the salivary gland. The common malignant tumors are NHL, squamous cell carcinoma (**Fig. 25.35**), renal cell carcinoma and malignant melanoma.

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CHAPTER 26

Thyroid

Chapter Contents

INTRODUCTION

Fine needle aspiration of cytology (FNAC) of thyroid has been reported long back in history. Martin and Ellis in New York in 1930 first time reported the usefulness of FNAC of thyroid.[1] Later on, FNAC of thyroid was reported by Söderström from Sweden.[2] In Karolinka, Sweden FNAC of thyroid lesions was routinely used and slowly it was popular in United State of America (USA) and other countries.

Fine needle aspiration of cytology is a very helpful and relatively noninvasive technique in the diagnosis of various thyroid lesions.[3] Any palpable thyroid nodule can be aspirated for diagnosis. The main aim of thyroid FNAC is to distinguish benign from malignant lesions and to determine the cases that require surgery. It may also be used in certain high-risk patients for follow-up. FNAC is a safe, cost effective and reliable technique. Due to introduction of this technique, the incidence of surgery in thyroid has been reduced considerably. The endocrine surgeons have widely accepted FNAC of thyroid. The common indications of FNAC of the thyroid lesions have been highlighted in **Box 26.1**.

BOX 26.1 **Indications of fine needle aspiration of cytology (FNAC) of thyroid**

- Any palpable nodule of thyroid
- Thyroid nodule of less than 10 mm diameter with clinical or suspicious features in USG
- Predominantly solid nodule in USG
- USG guided FNAC could be done in solid area of a cystic nodule and from a nonpalpable nodule

Abbreviations: USG, ultrasonography; FNAC, fine needle aspiration of cytology

BOX 26.2 **Clinical features suspicious for malignancy**

- Extreme age group
- Single dominant nodule
- Hoarseness of the voice
- History of irradiation of neck
- Family history of thyroid malignancy
- History of multiple endocrine neoplasia (MEN)
- Hard and fixed nodule
- Enlarged cervical lymph node

APPROACH TO FINE NEEDLE ASPIRATION OF CYTOLOGY OF THE THYROID

Clinical History and Physical Examination

Clinical details and physical findings of the patient are helpful in the interpretation of the cytology smear. Rapidly enlarged swelling, hoarseness of the voice, hard consistency and fixation with the deeper structure usually indicate malignancy (**Box 26.2**). History of irradiation or any history of carcinoma is needed.

A detailed family history of thyroid carcinoma is also helpful. Clinical features of hypothyroid or hyperthyroid should also be recorded.

Other Investigations

Following other investigations are preferable before doing FNAC:

- Thyroid stimulating hormone (TSH) level
- Thyroid scan
- Ultrasonography (USG) findings.

Before performing FNAC of the thyroid, serum TSH should be measured. If the patient has decreased serum TSH, then thyroid scan is recommended. All the cold nodules of thyroid should have FNAC. In case of normal or increased TSH level, an USG of the thyroid should be performed and USG guided FNAC should be done.

Techniques

The patient should be kept in supine position with a small pillow under his neck to make the thyroid region prominent. The technique of FNAC in the thyroid gland is essentially same. After cleaning the area of thyroid, the gland is hold in between the two fingers so that the thyroid will be prominent. Depending on the aspirator's choice, FNAC or nonsuction fine needle sampling (FNS) can be done. As the thyroid is a vascular organ so FNS is preferable in small swelling of the thyroid gland. However, in case of a cystic lesion, FNAC is preferable as there is a chance of spillage by FNS technique. It is recommended to do three to five passes of needle in each nodule.[4]

Multiple smears should be made immediately from the aspirated material and both air dried smear and alcohol fixed smears should be kept. If necessary, the material should be taken for cell block and other ancillary investigations.

The thyroid nodule is hold in between two fingers and gentle suction is done. FNS is preferable in thyroid as the material is free from blood.[5] Thyroid swelling is hold tightly in between the two fingers so that the thyroid will be prominent. Now, the needle is moved gently to and fro within the lesion. Material comes to the needle hub with the help of capillary pressure. The needle is gently withdrawn and the syringe is attached with the hub. The material in the needle is spread on the slide. The FNAC material can also be collected for cell block and flow cytometry to do ancillary technique.

FNAC can produce various preoperative artifacts. The common changes include infarction, hemorrhage, cystic degeneration, vascular proliferation, fibrosis and FNAC induced atypia (**Box 26.3**).[6] Linear artifactual changes occur along the needle track and this may be relatively easily identifiable.

Ultrasound Guidance Fine Needle Aspiration of Cytology

Recently, USG localization of thyroid lesions followed by USG guided FNAC of palpable thyroid nodule has been advocated to reduce the rate of the nondiagnostic material and subsequently to reduce the false negative interpretation.[7,8]

BOX 26.3 **Fine needle aspiration cytology (FNAC) induced changes in histology section**

- Infarction
- Hemorrhage
- Cystic degeneration
- Vascular proliferation
- Fibrosis
- Atypia

BOX 26.4 **Ultrasonography (USG) guided fine needle aspiration of cytology (FNAC) of thyroid**

Indications:

- Nonpalpable nodule
- Nodule with more than 25% cystic component
- Failure to get adequate material in prior aspiration

Advantages:

- Less nondiagnostic material
- Reduction of false negative cases
- Nodule of interest is aspirated
- Puncture of the critical structure can be avoided

In fact, USG guidance significantly helps in diagnosis and it gives proper guidance to target the palpable thyroid nodule. USG guided FNAC is not mandatory, but the pathologists should be encouraged to do USG guidance FNAC of thyroid lesions as far as possible. National Cancer Institute (NCI) sponsored Bethesda recommended USG guided FNAC in case of nonpalpable nodule or nodule that has more than 25% cystic component.[9] Other indication of USG guided FNAC is failure to get adequate material in prior aspiration (**Box 26.4**).

Complications

Fine needle aspiration of cytology of thyroid is almost free of any complications. Rarely needle tract seeding, hemorrhage, infection and recurrent laryngeal nerve injury have been described. These complications are mainly encountered if thick bore needle is used for FNAC.[10,11]

Optimal Material

Adequacy of the material in thyroid FNAC sample is a controversial issue. The various criteria have been proposed:

- Six groups of thyroid follicular cells with at least 10 cells in each group[12,13]
- Ten groups each with 20 or more cells[14]
- Six groups of cells on at least two aspirates[15]
- Eight groups on at least two slides.[16]

However, these criteria should not be strictly applied in thyroid FNAC because aspirates of colloid goiter may yield abundant colloid material with minimal number of cells. Cystic goiter may not yield thyroid follicular cells and ideally the aspiration should

be repeated from the solid area (if any) under USG guidance. FNAC smear should be considered as suboptimal in case of extensive drying artifact, blood or thick ill-spread.

ANATOMY AND HISTOLOGY

The thyroid glands are located in the anterior part of neck in front of trachea and inferior to larynx. The gland is composed of two lobes, which are connected by central part known as isthmus. The thyroid gland is covered by thick fibrous septae. The fibroconnective tissue has divided the lobes into multiple smaller lobules.

Follicles are the basic structural unit of thyroid gland. Each follicle contains homogenous eosinophilic colloid material. Follicles are lined by low cuboidal to columnar cells. Thyroid follicular cells liberate tri-iodothyronine (T_3) and thyroxine (T_4) hormone. Due to some unknown reason, thyroid follicular cell may undergo Hurthle cell change. These are large cells with abundant eosinophilic cytoplasm and central round nuclei. Electron microscopy of such cells shows abundant mitochondria.

Parafollicular cells are present in the periphery of the follicles as small cluster. These cells are three to four times larger than the follicular cells. They have pale moderate amount of cytoplasm with central spherical nuclei. Parafollicular cells secrete calcitonin hormone that takes important role in calcium homeostasis. Calcitonin hormone inhibits bone resorption by osteoclasts and, thereby helps to lower the calcium concentrations in blood.

Diagnostic Accuracy

It is difficult to assess the sensitivity and specificity of FNAC of thyroid because vast majority of benign lesions of thyroid are not undergone any surgery. Moreover, only selected patients with clinical symptoms and signs are operated.

False negative rate of thyroid FNAC varies from 1% to 11%.[17,18] False negative report of cytology is mainly due to inadequate sampling of thyroid. The false positive rate of FNAC of thyroid is about 3% and it largely depends on diagnostic category.[19] The major causes of false positive cytology are hyalinizing trabecular adenoma and follicular neoplasia. The error in FNAC of thyroid depends both on sampling error and interpretation error. Overall sensitivity and specificity of thyroid FNAC is 83% and 92%, respectively.[20,21]

In a large meta-analysis study by Tee et al., the sensitivity and specificity of FNAC of thyroid were measured by a statistically designed model excluding the significant bias in previous study designs. Only those studies were selected that have histopathological outcome in every case. They noted that the sensitivity of FNA may be as low as 66%. It was also noted that the specificity of FNA has a consistently high value of 93%.[22] The authors concluded that FNAC may miss a significant fraction of patients with thyroid malignancy. All the patients should be followed up either conservatively or with repeat FNAC biopsy to exclude malignancy.

Terminology

There are various reporting terminologies of FNAC smear of thyroid lesions. Papanicolaou Society of Cytopathology Task Force on Standards of Practice, 1997, recommended following terminology system:[23]

- Inadequate or unsatisfactory
- Benign
- Atypical cells present
- Suspicious for malignancy
- Malignant.

However, NCI sponsored the conference on thyroid FNAC on October 22–23, 2007 in Bethesda[24] and recommended this updated terminology:

- Benign lesions: Colloid goiter, lymphocytic thyroiditis (LT), hyperplastic nodule, etc.
  - Risk of malignancy: Less than 1%
  - Management: Clinical follow-up
- Follicular lesions of undetermined significance or atypia of undetermined significance: The smear pattern is not convincingly either benign or follicular neoplasm (FN).
  - Risk of malignancy: 5–10% risk of malignancy
  - Management: Repeat FNAC should be done along with clinical and radiological correlation
- Follicular neoplasm or suspicious of FN: Follicular and Hurthle cell neoplasm. The other diagnostic terms in this category include follicular lesions, micropapillary proliferation and suggestive of neoplasm.
  - Risk of malignancy: 20–30%
  - Management: The patients should have definitive surgery such as lobectomy or hemithyroidectomy
- Suspicious for malignancy: Suspicious for follicular variant of papillary carcinoma of thyroid (FVPCT), medullary carcinoma (MC), certain cases of anaplastic carcinoma of thyroid, non-Hodgkin lymphoma (NHL) and metastatic carcinoma.
  - Risk of malignancy: 50–75% risk of malignancy
  - Management: Thyroidectomy is advised
- Malignancy: Specify the type of malignancy
  - Risk of malignancy: 97–99% risk of malignancy
  - Management: Thyroidectomy is advised
- Inadequate: Here, the specimen is inadequate due to
  - Low cellularity
  - Lack of follicular cells
  - Poor fixation

Management: Repeat FNAC is advised.

Normal Aspirated Material

- *Colloid* (**Fig. 26.1**): Gross appearance of colloid is thick to thin brownish material. The MGG stained cytology smear shows a pale blue acellular material.
- *Thyroid follicular cells* (**Figs 26.2A and B**): Follicular cells are present in small clusters and follicles. The cells are round with scanty cytoplasm and round monomorphic nucleus. Nuclear chromatin is homogeneously spread.
- *Foam cells* (**Fig. 26.3**): The foam cells show abundant foamy cytoplasm with small monomorphic nucleus.

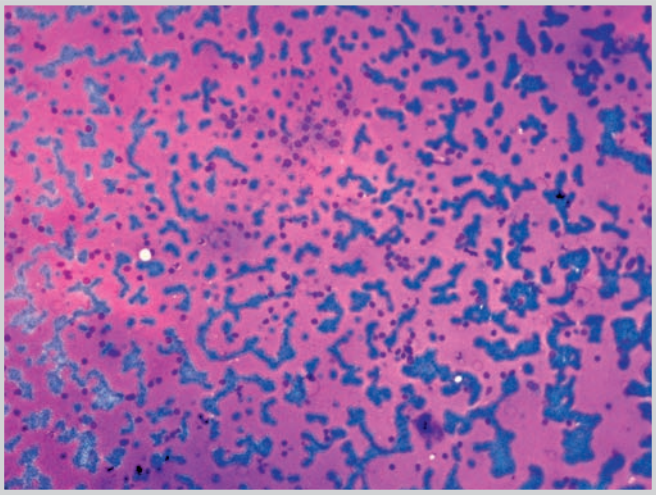


Fig. 26.1: Pale blue thin colloid material [May-Grünwald-Giemsa (MGG) × medium power (MP)]

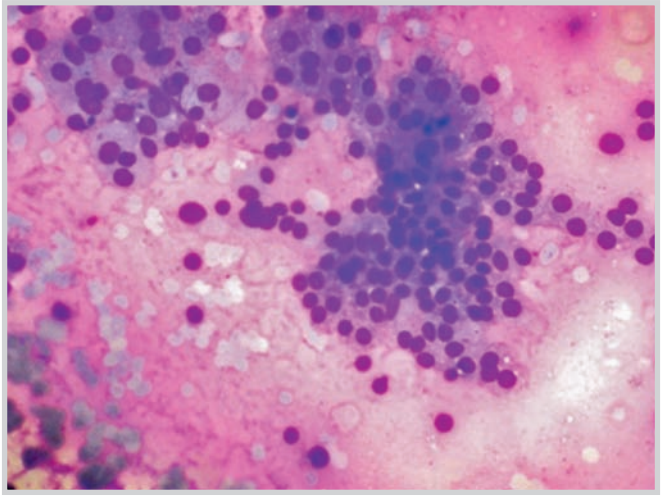


Fig. 26.2B: Small cluster of thyroid follicular cells with round monomorphic nuclei and moderate amount of cytoplasm (MGG × HP)

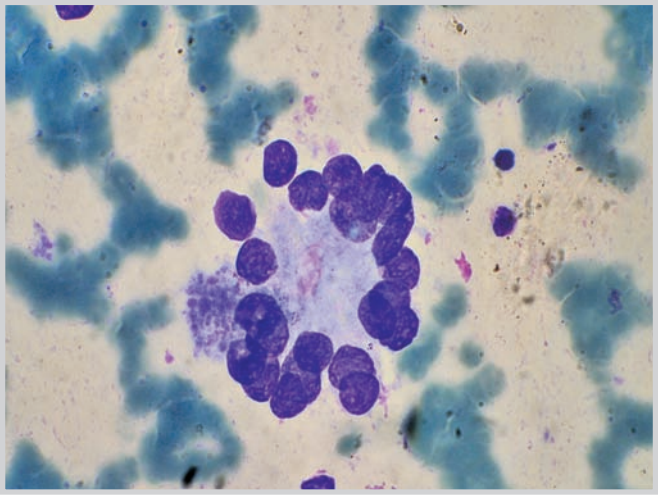


Fig. 26.2A: Thyroid follicle lined by round monomorphic follicular cells [MGG × high power (HP)]

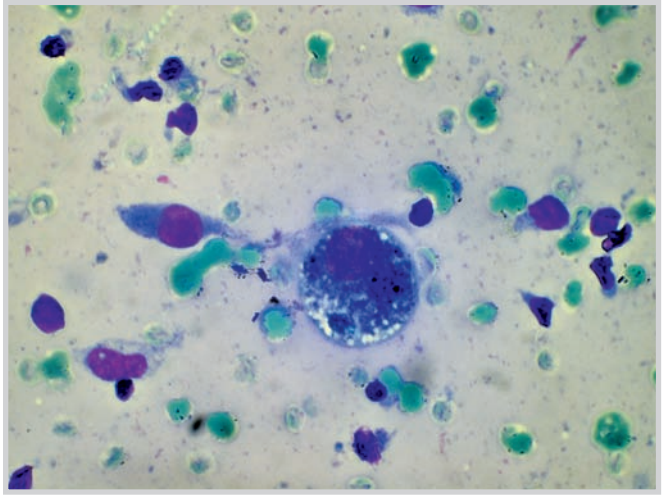


Fig. 26.3: Cytology smear of foam cell showing abundant foamy cytoplasm with phagocytosed material in the cytoplasm (MGG × HP)

DISEASES OF THYROID

The classification of commonly encountered lesions on FNAC of the thyroid has been given in **Table 26.1**.

TABLE 26.1: Classification of thyroid lesion

Non-neoplastic	*Neoplastic*
Colloid goiter	Follicular tumor
Thyroiditis	Follicular adenoma
Acute	Follicular carcinoma
Chronic	Hurthle cell tumor
Sub-acute	Hurthle cell adenoma
Riedel's struma	Hurthle cell carcinoma
	Papillary carcinoma
	Medullary carcinoma
	Anaplastic carcinoma
	Insular carcinoma
	Non-Hodgkin lymphoma
	Sarcoma
	Metastatic carcinoma

Non-neoplastic

Colloid Goiter

Nodular colloid goiter is the commonest diseases of thyroid. The patients have iodine deficiency due to low iodine in water and soil. The deficient iodine leads to decrease production of thyroid hormone from thyroid gland. The deficient thyroid hormone induces stimulation of TSH secretion from pituitary gland. Excess TSH hormone causes thyroid follicular hyperplasia with massive storage of colloid. The thyroid may have single or

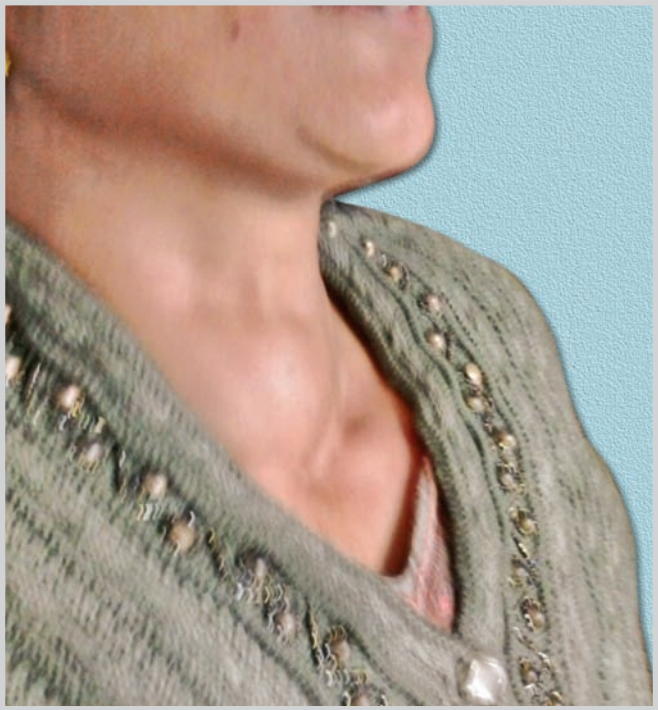


Fig. 26.4: Nodular goiter in a young female

BOX 25.5 Cytology of nodular goiter

- Abundant colloid
- Discrete or flat sheet of thyroid follicular cells
- Macrophages

multiple nodules (**Fig. 26.4**). Uncommonly, the thyroid may be massively enlarged causing tracheal obstruction. Salient cytological features are highlighted in **Box 26.5**.

Cytology *(***Figs 26.5A and B***)*: The aspirate yields thick to thin brownish material on gross examination. In May-Grünwald-Giemsa (MGG) stained smear, the colloid material looks like pale blue to dark blue material. In Papanicolaou's stained smear, it is difficult to appreciate water-clear colloid and it looks like pink homogenous material. Varying amount of thyroid follicular cells is embedded in the colloid. The cells are present in flat small sheets, in small clusters and discretely. The individual cells are small cuboidal with round with monomorphic nuclei and fine chromatin. Many follicular cells are naked with stripped of cytoplasm. These cells may resemble lymphocytes. Nodular goiter often shows cystic degeneration and in that case the smears show abundant foamy macrophages containing dark brown hemosiderin laden pigments. Longstanding goiter may show multinucleated giant cells due to reaction of colloid. Occasionally, fibrosis occurs in the nodular goiter and the smear may show young active fibroblasts. The large atypical active fibroblast may be misinterpreted as malignant cell.

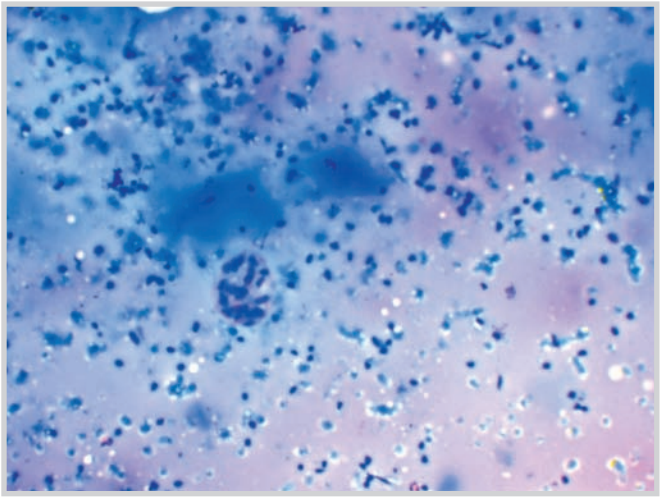


Fig. 26.5A: Abundant thick and thin colloid along with scattered benign thyroid follicular cells in colloid goiter (MGG × MP)

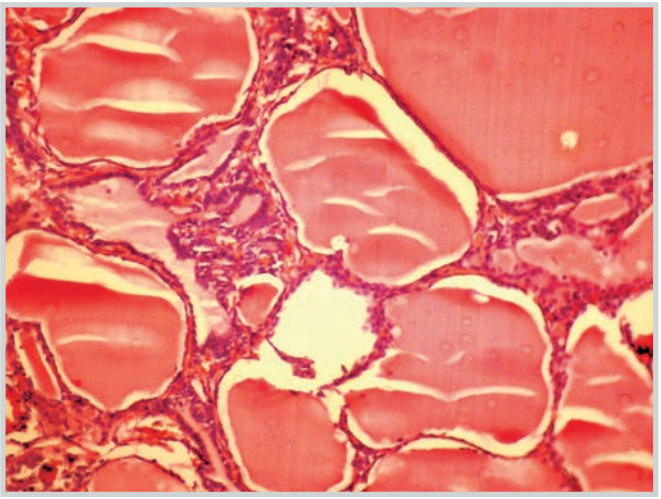


Fig. 26.5B: Histopathology of the same showing variable sized thyroid follicles filled with colloid [Hematoxylin and Eosin (H & E) × MP]

Differential Diagnosis

- Colloid goiter versus hyperplastic nodules: At times, the amount of thyroid follicular cells may be relatively more and there may be difficulties to differentiate a colloid goiter from a hyperplastic or adenomatoid nodule. The relative excess of colloid is a helpful diagnostic feature of colloid goiter
- Atypical large cells

Adenomatoid Nodule or Hyperplastic Nodule

This is mainly a cytological terminology. Smears show good number of thyroid follicular cells and scanty colloid. The follicular cells are arranged in flat sheet, macrofollicles, microfollicles and singly (**Box 26.6**). The follicular cells are monomorphic with regular nuclear margin. However, occasional cells may show

BOX 26.6 Adenomatoid hyperplasia or hyperplastic nodules

- Cellular smear
- Dissociated and microfollicular arrangement
- Hurthle cells
- Foamy macrophages
- Scanty colloid

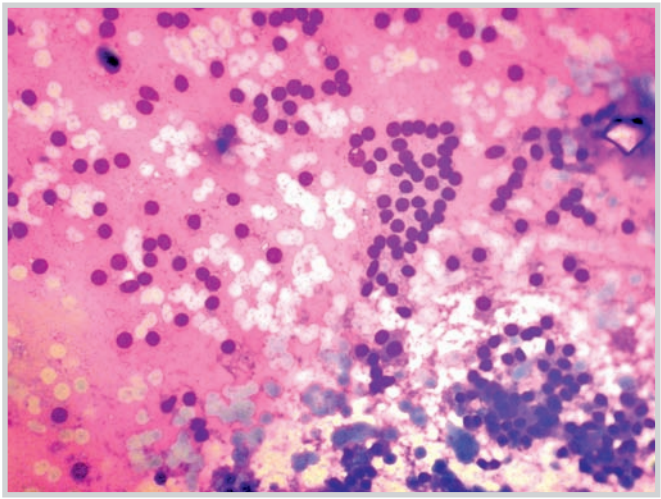


Fig. 26.6: Multiple follicles and thin colloid in adenomatoid nodule (MGG × MP)

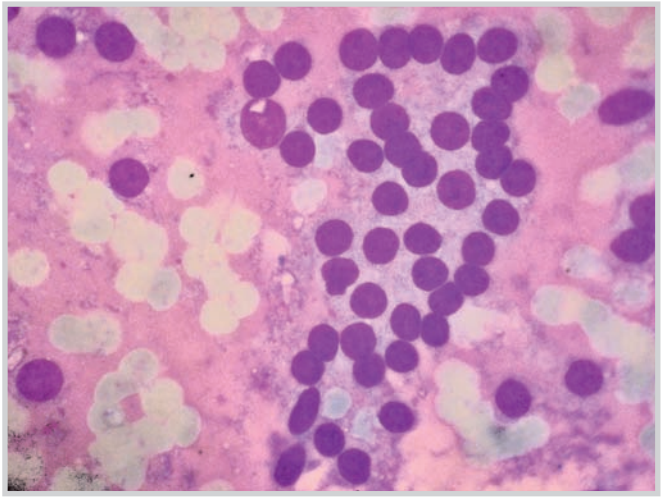


Fig. 26.7: Higher magnification showing microfollicles and colloid in adenomatoid nodule (MGG × HP)

nuclear atypia. Hurthle cells and foamy macrophages may be seen in the background. At times, the microfollicles may be relatively abundant and the differentiation of hyperplastic nodules versus FN is very difficult (**Figs 26.6** and **26.7**). The neoplastic lesions are usually cold nodule. There may be variable types of cells such as follicular cells, Hurthle cells and foam cells in adenomatoid nodule.

- *Diagnostic difficulties*: Follicular neoplasm

Diffuse Toxic Goiter (Grave's Disease)

This is an autoimmune disorder characterized by diffuse enlargement of thyroid along with features of toxicity such as tachycardia, exophthalmos, weakness, weight loss, dermatopathy and irritability. The patients have immunoglobulin G (IgG) and thyroid stimulating immunoglobulin (TSI) directed against the TSH receptor of the thyroid follicular cells. The antibody functions as agonist of TSH receptors and stimulates thyroid hormone synthesis. FNAC is not indicated in Grave's disease except the nodular swelling in thyroid.

Cytology (Figs 26.8A and B)

Aspirates of the Grave's disease show good amount of cells and scanty colloid (**Box 26.7**). The follicular cells are in clusters and scattered. The cells have moderate to abundant eosinophilic cytoplasm and centrally placed round nuclei with prominent nucleoli. Nuclear atypia is often noted particularly long standing Grave's disease treated medically. The nuclei may show nuclear grooving and chromatin clearing simulating papillary carcinoma of thyroid (PCT). Cluster of cells with peripheral red granules known as fire flare appearance is a common findings.[25]

Thyroiditis

Acute Thyroiditis

Acute thyroiditis patient presents with fever, tachycardia, and sudden painful enlargement of thyroid. The thyroid is diffusely enlarged and tender on palpation. FNAC is rarely done in acute thyroiditis.

Cytology: Smears show abundant acute inflammatory cells consisting of polymorphs, lymphocytes and necrotic tissue fragments (**Box 26.8**). Scattered degenerated thyroid follicular cells may also be seen on the background. The follicular cells may exhibit reparative changes and nuclear atypia. Necrosis and polymorphs infiltration are also seen in anaplastic carcinoma of thyroid. A careful examination is needed to exclude the possibility of anaplastic carcinoma. If required, a repeat FNAC should be done from multiple sites of thyroid.

Differential diagnosis: Anaplastic carcinoma.

Subacute Granulomatous Thyroiditis

This is probably related with viral infection. The patient is usually a young woman and presents with painful swelling of thyroid, fever and myalgia, sore throat and high erythrocyte sedimentation rate. The thyroid is diffusely enlarged. In the initial phase of the disease, the patient may have hyperthyroidism. This disease is usually self-limiting. Subacute thyroiditis may be mistaken as neoplasm due to firm thyroid swelling.

Cytology *(***Fig. 26.9***)*: Destruction of thyroid follicular releases colloid in the neighboring tissue causing granulomatous reaction. The FNAC smears show many multinucleated giant

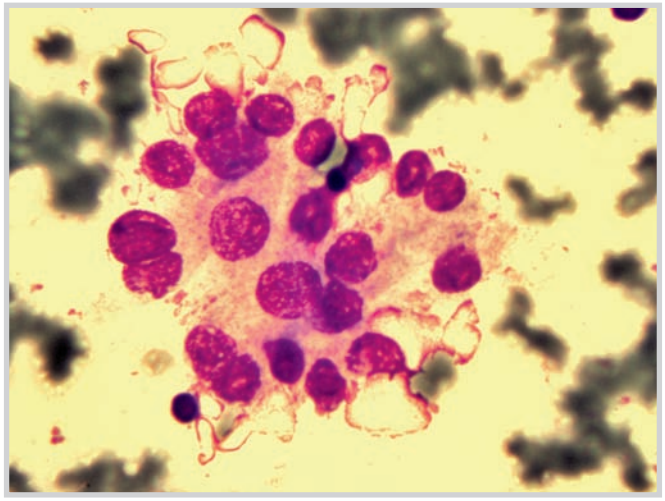


Fig. 26.8A: Fire flares appearance in Grave's disease of thyroid (MGG × HP)

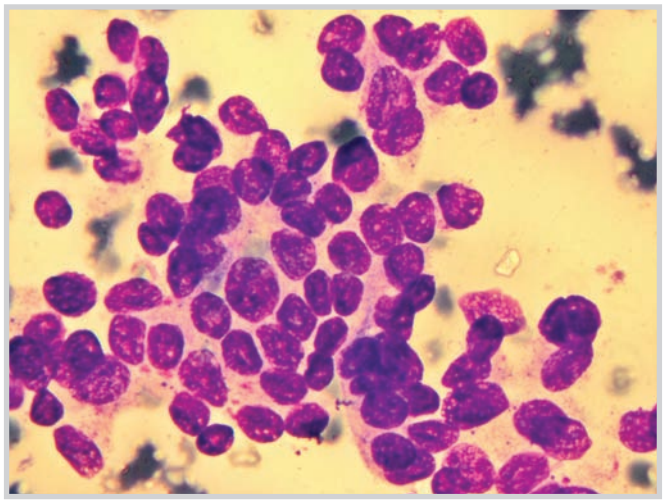


Fig. 26.8B: Grave's disease of thyroid: Nuclear enlargement and pleomorphism along with occasional nuclear grove (MGG × HP)

BOX 26.7 Diffuse toxic goiter (Grave's disease)

- Cellular
- Scanty watery colloid
- Enlarged cell with vacuolated cytoplasm
- Prominent nucleoli
- Fire flares appearance

BOX 26.8 Acute thyroiditis

- Polymorphs and lymphocytes
- Necrosis
- Degenerated thyroid follicular cells.

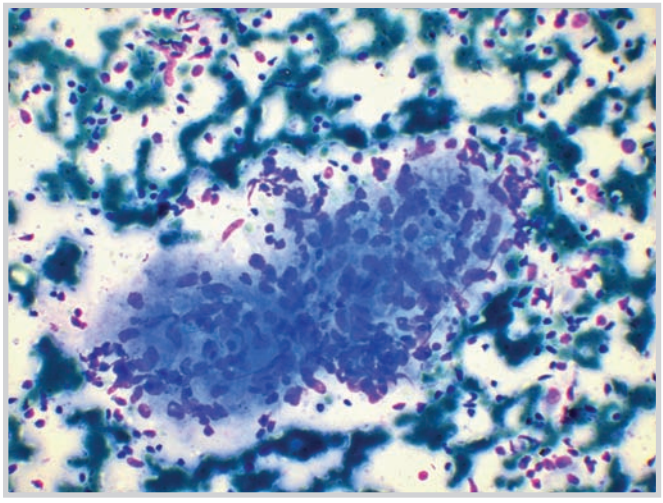


Fig. 26.9: Epithelioid cell granuloma and reactive lymphoid cells in subacute granulomatous thyroiditis (MGG × MP)

BOX 26.9 Subacute granulomatous thyroiditis

- Dirty background formed by cellular debris and scanty colloid
- Epithelioid cell granulomas
- Multinucleated giant cells
- Lymphocytes and plasma cells
- Benign thyroid follicular cells with paravacuolar granulations

cells (**Box 26.9**). These cells are large with 50–100 nuclei. Ill-formed epithelioid cell granulomas along with polymorphs and lymphocyte and plasma cells are also noted. Background of the smear shows cellular debris and colloid. In addition, clusters and discrete thyroid follicular cells with prominent intracytoplasmic paravacuolar granules of lipofuscin and lysosomal debris are seen.

The presence of multinucleated giant cells is an important finding in granulomatous thyroiditis. However, similar cells are also noted in LT and PCT. It is important to mention that in early or late stage of subacute thyroiditis, the typical cytological features may be absent.

Differential Diagnosis

- Chronic LT
- Tuberculosis: It shows almost similar features. However, tuberculosis is very uncommon in thyroid.

Chronic Lymphocytic Thyroiditis (Hashimoto's Thyroiditis)

Chronic LT is an autoimmune thyroiditis and is much more common in women than men. It can occur in any age group; however, it is more common in fourth to six decade. Thyroid antimicrosomal antibody and antithyroglobulin antibodies

are elevated in the patient and destroy the thyroid follicle. The damage of thyroid gland may release thyroid hormone in early phase causing hyperthyroidism. Later on, the patient goes to hypothyroid phase.

Cytology (Figs 26.10 to 26.18)

In early stage of LT, the thyroid gland is infiltrated by florid reactive lymphoid cells. There is little destruction of thyroid follicles and the patients are usually euthyroid. In the advanced stage of LT, there is considerable destruction of thyroid follicles by lymphoid cells and the patients are mostly hypothyroid in this phase (**Box 26.10**). In the initial phase of LT, the aspirate shows florid germinal center cells consisting of immunoblasts, small-cleaved and large-cleaved mature lymphocytes and plasma cells. Occasional clusters of thyroid follicular cells are noted in the background. The smears may be mistaken as reactive lymph node if the follicular cells are overlooked.

In the advanced or late stage, there is too some extent fibrosis of the gland and the smear shows sparse lymphocytes associated with adjacent follicular cells. There is infiltration of lymphoid cells within the follicles and evidence of destruction of thyroid follicles.[26,27] In addition, the smears also show Hurthle cells. These cells are present in loose clusters. The Hurthle cells have abundant eosinophilic cytoplasm with centrally placed regular nuclei. Nuclei may show crowding and overlapping. At times, considerable nuclear pleomorphism of the Hurthle cells may give false impression of malignancy, particularly Hurtle cell neoplasm.

The smears of LT show scanty colloid. There may be ill-formed epithelioid granulomas, multinucleated giant cells and foamy histiocytes in the aspirate. Epithelioid cell granulomas may give rise to the differential diagnosis of subacute granulomatous thyroiditis. However, the clinical findings of painful swelling of thyroid, fever and myalgia along with multiple giant cells and epithelioid cell granulomas favor subacute granulomatous thyroiditis.

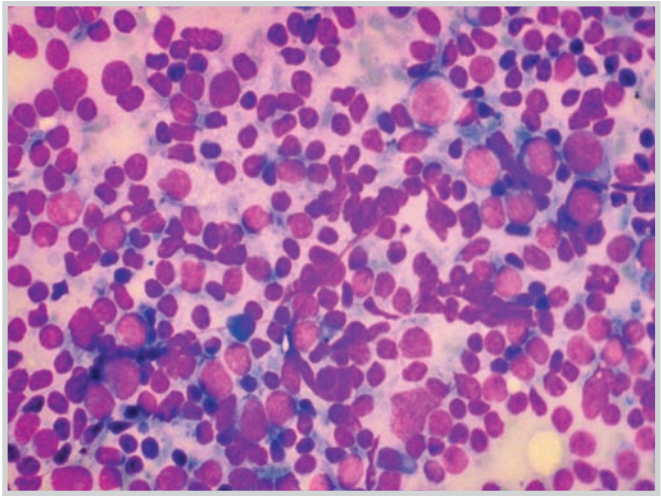


Fig. 26.10: Abundant reactive lymphoid cells in lymphocytic thyroiditis (MGG × HP)

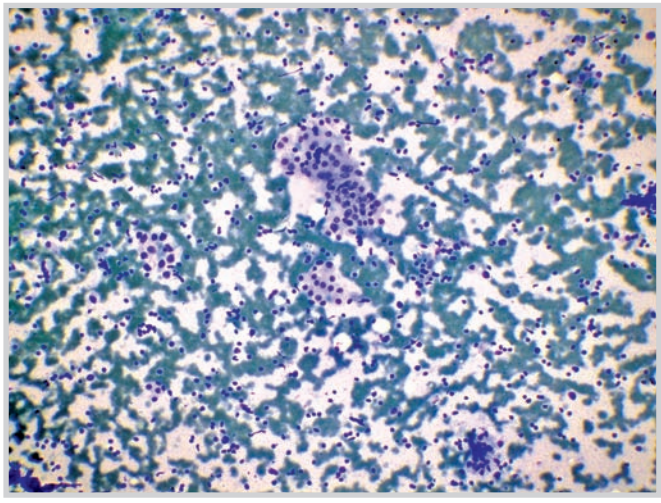


Fig. 26.11: Excess reactive lymphoid cells in the background along with the thyroid follicles in lymphocytic thyroiditis [MGG × low pressure (LP)]

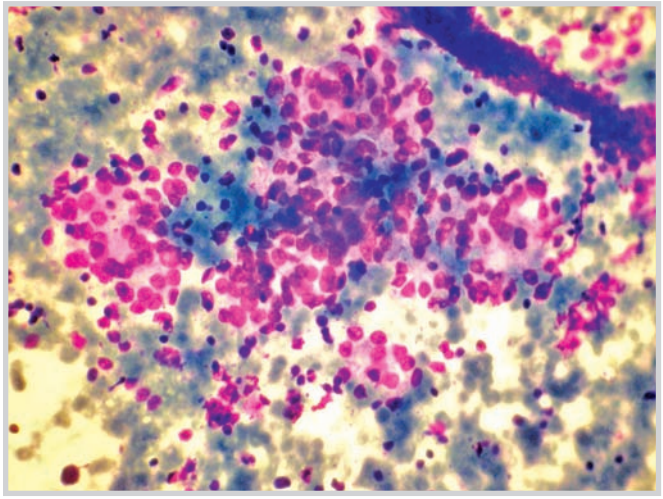


Fig. 26.12: Lymphoid cells infiltration in the thyroid follicular cells in lymphocytic thyroiditis (MGG × HP)

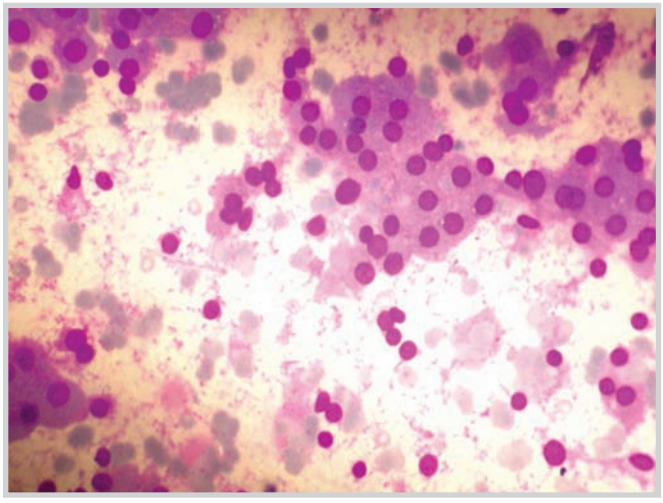


Fig. 26.13: Hurthle cell changes in lymphocytic thyroiditis (MGG × MP)

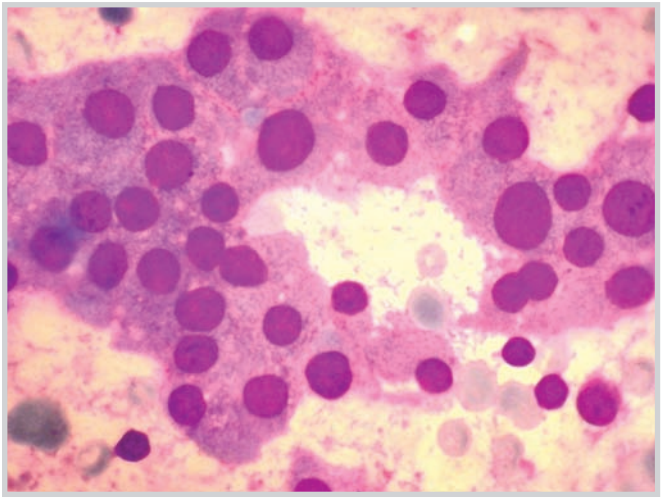


Fig. 26.14: Hurthle cell changes in higher magnification in lymphocytic thyroiditis (MGG × HP)

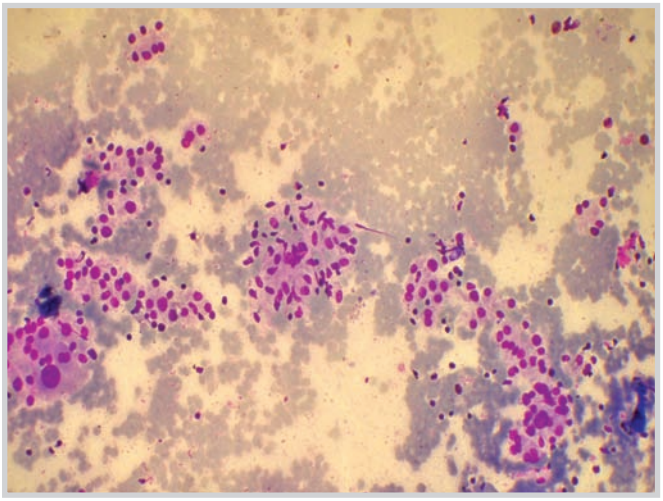


Fig. 26.15: Ill-formed epithelioid cell granuloma in lymphocytic thyroiditis (MGG × MP)

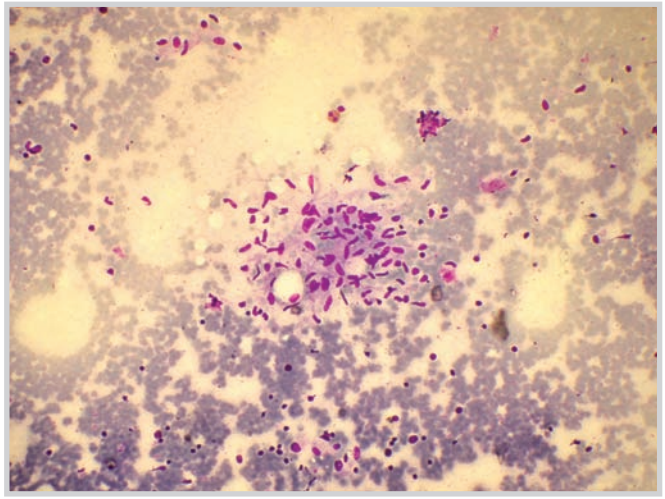


Fig. 26.16: Higher magnification of granulomas in lymphocytic thyroiditis (MGG × HP)

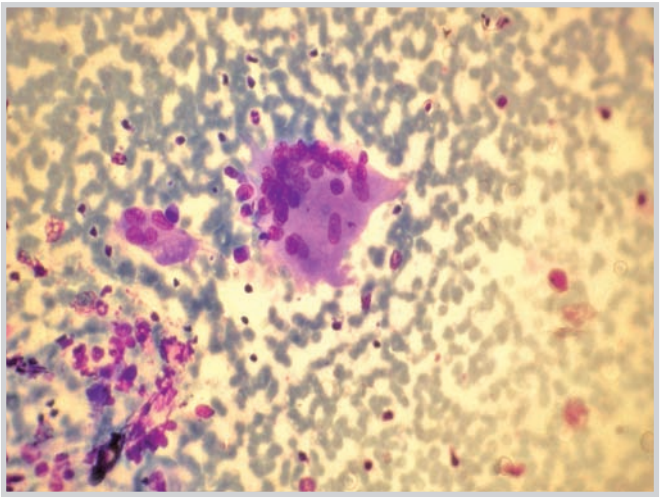


Fig. 26.17: Multinucleated giant cells in lymphocytic thyroiditis (MGG × MP)

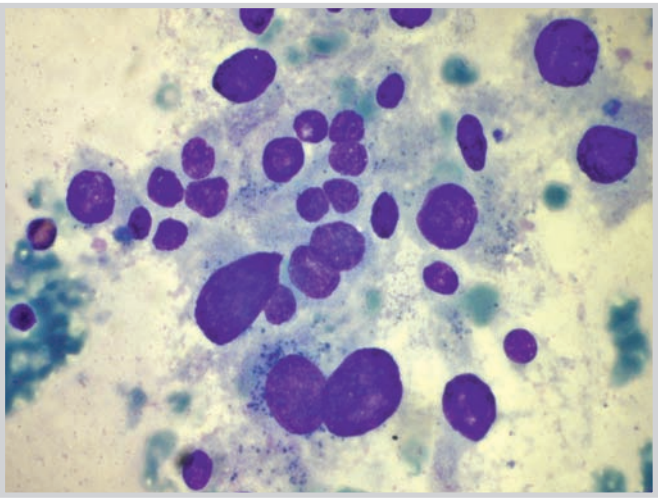


Fig. 26.18: Marked nuclear enlargement and pleomorphism in lymphocytic thyroiditis (MGG × HP)

BOX 26.10 Chronic lymphocytic thyroiditis

- Lymphocytic infiltration in thyroid follicular cells
- Lymphocytes and plasma cells
- Scanty colloid
- Hurthle cells
- Multinucleated giant cells
- Ill-formed epithelioid cell granuloma
- Elevated thyroid antimicrosomal antibody and antithyroglobulin antibodies.

Diagnostic Difficulties

- Hurthle cell neoplasm: Described below
- Non-Hodgkin's lymphoma: Florid LT should be distinguished from NHL particularly low-grade lymphoma of thyroid. Demonstration of light chain restriction in B-NHL or aberrant expression of T-cell markers in T-NHL on flow cytometry indicates monoclonality and suggests the possibility of NHL
- Subacute granulomatous thyroiditis

Neoplastic

Follicular Neoplasm

Thyroid nodule with predominant follicular pattern in the smear is the diagnostic challenge to the cytopathologist. The various benign and malignant entities of thyroid may simulate this pattern. This includes nodular hyperplasia, follicular adenoma (FA), follicular carcinoma (FCa) and FVPCT. Differentiating the cases of FA from FCa in FNAC smear is not possible. Probably, FNAC just helps to identify the nodule that needs surgical excision. FA is a well-encapsulated benign neoplasm with predominant follicular cell differentiation. The tumor does not show any capsular or vascular invasion and follicular cells do not have the nuclear characteristics of papillary carcinoma. FA is a monoclonal tumor and commonly affects female (female: male ratio is 3:1). The tumor presents as a solitary well-encapsulated nodule with variant histological pattern such as normofollicular, macrofollicular, microfollicular and trabecular or solid.

Follicular carcinoma of thyroid represents 10–15% of the thyroid cancers. They show typical follicular differentiation, lack of nuclear features of PCT and capsular or vascular invasion. Capsular and vascular invasion are the two most important characteristics of FCa. Depending on the extent of such invasion FCa is divided into:

1. Minimally invasive FCa
2. Invasive FCa

Minimally invasive FCa is a well-encapsulated tumor with partial infiltration of tumor cells in the capsule. In case of invasive FCa, the tumor extends entirely through the capsule and shows vascular invasion. The surrounding soft tissue may also be involved in invasive FCa.

Cytology (Figs 26.19 to 26.22)

FNAC smears show abundant cellularity with scanty colloid (**Box 26.11**). The cells are arranged in three-dimensional crowded groups, microfollicles and trabeculae.[28] Colloid is thin and often present focally as dense droplets. The less the amount of colloid, more should be the suspicion for FN. Macrofollicles always breakdown in small fragments in the aspirated material. However, architectural pattern of microfollicles are usually preserved in FNAC and its presence suggests the diagnosis of FN. In FNAC material, microfollicles are defined as small follicles composed of near about 15 cells and at least two-third of the circumference of them are covered by follicular cells.[29] Microfollicular pattern are also demonstrated in the hyperplastic nodular goiter and therefore the smear of FN should be carefully interpreted. In fact, as mentioned before colloid in FN is denser than that of nodular hyperplastic goiter. FN shows significant proportion of microfollicles with nuclear crowding and overlapping. Aspirate may show different types of changes such as:

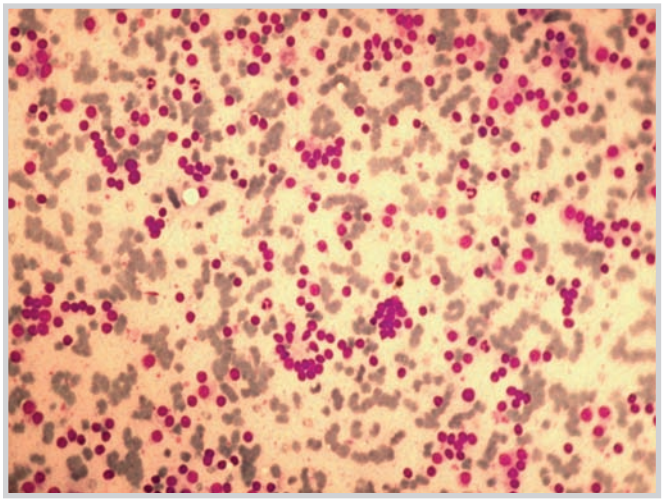


Fig. 26.19: Abundant discrete cells and follicles in follicular neoplasm (MGG × LP)

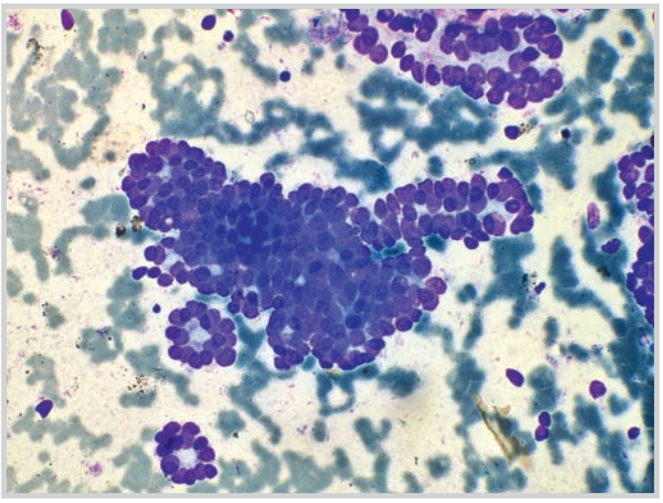


Fig. 26.20: Multiple microfollicles and solid sheets of cells in follicular neoplasm (MGG × MP)

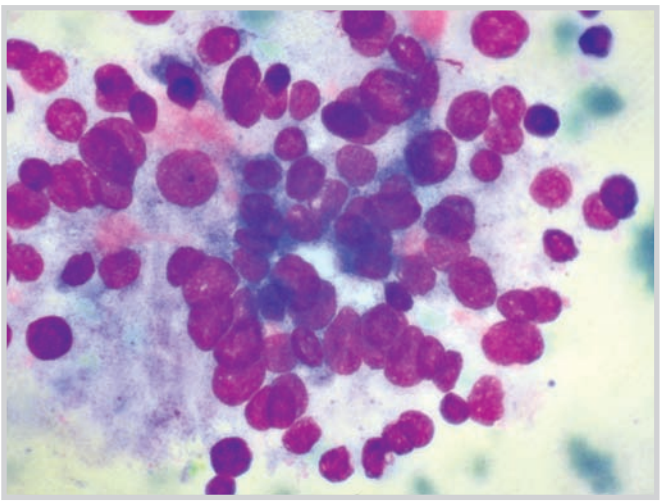


Fig. 26.21: Higher magnification showing microfollicles in follicular neoplasm of thyroid [MGG × oil immersion (OI)]

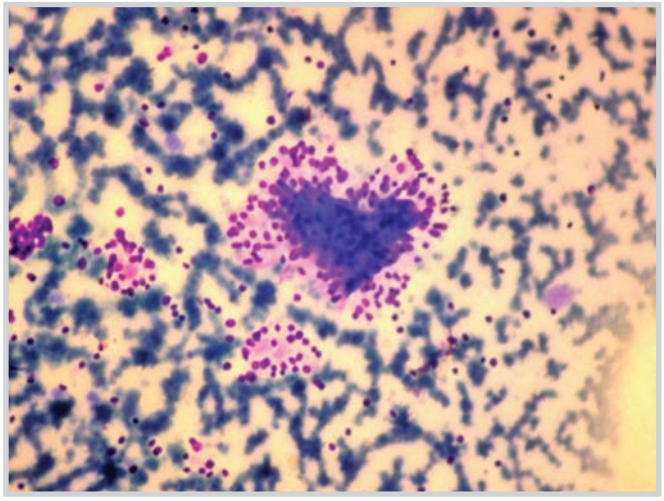


Fig. 26.22A: Multiple clusters and microfollicles in osteolytic lesion of shaft of the humerous in a case of follicular carcinoma (MGG × LP)

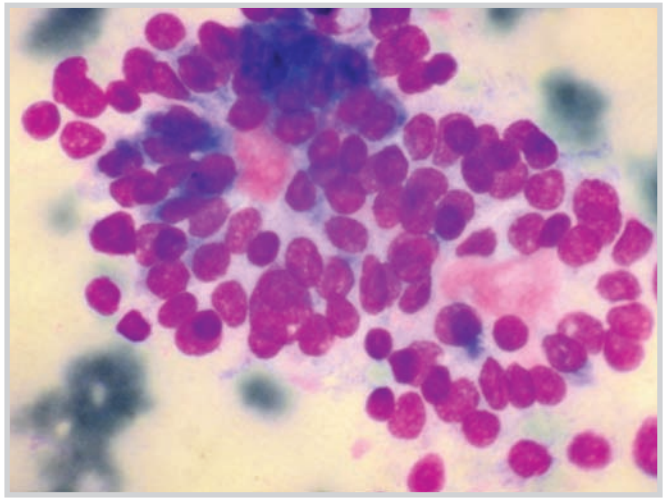


Fig. 26.22B: Higher magnification of the same showing microfollicles formed by monomorphic follicular cells (MGG × LP)

BOX 26.11 Follicular neoplasm

- Abundant cellularity
- Scanty droplet like dense colloid
- Microfollicles
- Three-dimensional crowded groups
- Trabeculae
- Cold nodule on scan
- Usually solitary nodule

- Cells with bizarre nuclei
- Prominent Hurthle cell change
- Papillary structures
- Signet ring cells

It is truly not possible to differentiate FA and carcinoma on FNAC smears. However, high nucleocytoplasmic ratio, nuclear atypia and coarse granular chromatin are suggestive of FCa.[23,28,30]

Without proper clinical history, aspirates of parathyroid adenoma can be mistakenly considered as FN. On cytomorphology alone, it is very difficult to diagnose parathyroid adenoma. However, absence of colloid, abundant clear cells and history of hypercalcemia should raise the suspicion of parathyroid adenoma. In doubtful situation, immunocytochemistry may be helpful to confirm the diagnosis parathyroid adenoma. The cells of parathyroid adenomas are thyroglobulin negative, parathormone positive, chromogranin positive.

Follicular variant of papillary carcinoma also shows multiple microfollicles and may pose diagnostic problem with follicular neoplasm. The diagnostic clues of follicular variant of papillary carcinoma in aspirates are chewing gum like colloid, nuclear grooving and intranuclear pseudoinclusion (**Table 26.2**).[31]

Diagnostic Difficulties

- Follicular hyperplasia versus neoplasm
- Follicular adenoma versus carcinoma
- Parathyroid adenoma
- Follicular variant of papillary carcinoma

TABLE 26.2: Distinguishing features of follicular neoplasm and follicular variant of papillary carcinoma

Features	*Follicular neoplasm*	*Follicular variant of papillary carcinoma*
Colloid	Scanty dense droplet-like	Thick ropy colloid
Thyroid cells arrangement	Abundant microfollicles, 3D clusters, trabeculae	Microfollicles and discrete cells
Nuclear grooving	Absent	Present
Chromatin	Evenly dispersed fine nuclear chromatin in adenoma to coarse chromatin	Fine powdery chromatin; nuclear clearing present
Intranuclear inclusion	Absent	Present
Psammoma body and multinucleated giant cells	Absent	Present
RET-PTC oncogenic transformation	Absent	Present

Abbreviations: 3D, three-dimensional; RET, rearranged intransformation; PTC, papillary thyroid carcinoma

Hurthle Cell Neoplasm

Hurthle cell tumor as a distinct separate entity is controversial. World Health Organization (WHO) recognized this entity only as oncocytic subset of follicular neoplasms.[32]

Hurthle cell tumor represents 3–5% of all thyroid tumors and is classified as Hurthle cell adenoma and carcinoma. The mean age of the patient is about 50 years. The same histological criteria of FCa are also applied for Hurthle cell carcinoma. The term oncocytes are preferable than "Hurthle cell". These are large cells with abundant deeply eosinophilic granular cytoplasm. This granularity of the cytoplasm is due to accumulation of mitochondria. The oncocytes are positive for thyroglobulin, cytokeratin-14 (CK14) and glucose transporter-4 (GLUT-4).[33-35]

Cytology (Figs 26.23 to 26.26)

Aspirates of Hurthle cell neoplasm show abundant dissociated oncocytes (**Box 26.12**). The cells are polygonal, large with abundant eosinophilic cytoplasm having centrally placed enlarged nuclei. The nuclei of the cell show prominent nucleoli and macronucleoli. The Hurthle cells are polygonal, large cells with abundant eosinophilic granular cytoplasm.[36,37] The nuclei are eccentric in position, enlarged with prominent nucleoli (**Box 26.13**). Variable degree of cellular atypia may be noted in Hurthle cells in the form of nuclear enlargement, binucleation, and multinucleation. Colloid is scanty to absent. Chronic LT may often show marked Hurthle cell changes. Markedly pleomorphic Hurthle cells with background lymphocytes should always raise the possibility of LT (**Table 26.3**). It has been suggested that intracytoplasmic lumen and transgressing vessels are also diagnostic features of Hurthle cell neoplasm.[38] Hurthle cell neoplasm often mimic as MC because both the tumors show dissociated cells with plasmacytoid appearance. However, the cells of MC show reddish granular cytoplasm and they usually do not have prominent nucleoli (**Table 26.4**). Moreover, Hurthle cell neoplasm is negative for calcitonin stain.

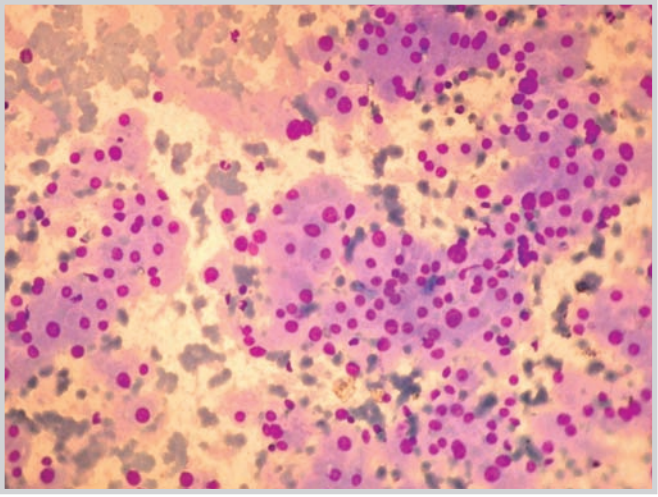


Fig. 26.23: Abundant discrete Hurthle cells in Hurthle cell neoplasm of thyroid (MGG × MP).

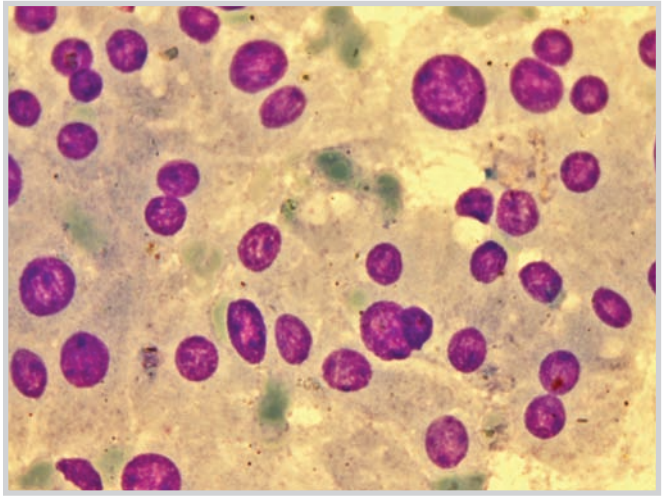


Fig. 26.25: Hurthle cells in higher magnification (MGG × OI)

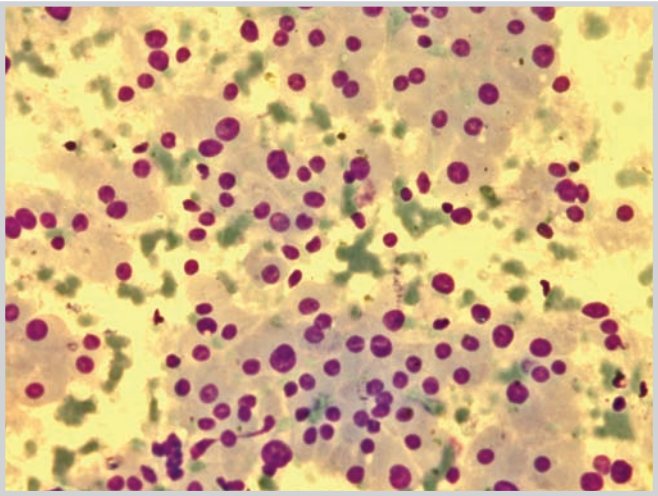


Fig. 26.24: Large cells with abundant eosinophilic granular cytoplasm in Hurthle cell neoplasm of thyroid (MGG × HP)

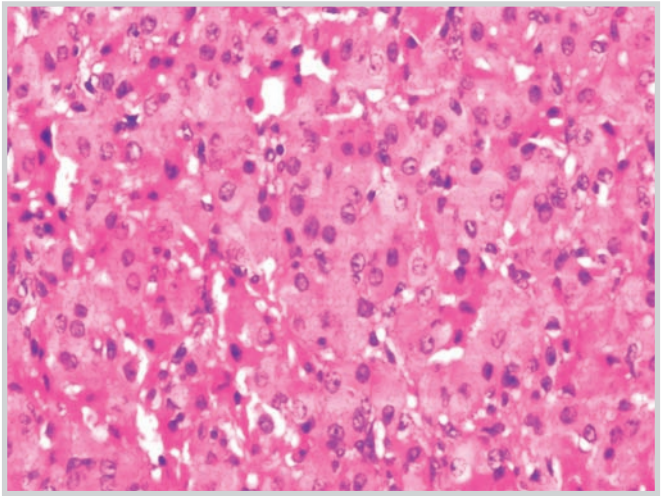


Fig. 26.26: Histopathology of the above case of Hurthle cell adenoma (H & E × MP)

BOX 26.12 Oncocytes (Hurthle cell) in thyroid

- Large cells with abundant deeply eosinophilic granular cytoplasm
- Enlarged round to oval nucleus with a prominent nucleolus
- Rich in mitochondria
- Positive for GLUT-4, CK14, thyroglobulin

Noted in:

- Hurthle cell neoplasm
- LT
- Nodular goiter

Abbreviations: GLUT-4, glucose transporter-4; CK14, cytokeratin-14; LT, lymphocytic thyroiditis

BOX 26.13

- Multiple clusters and dissociated Hurthle cells
- Enlarged nuclei with prominent macronucleoli
- Colloid is scanty to absent

TABLE 26.3: Differential diagnosis of Hurthle cell tumor and lymphocytic thyroiditis

Features	*Hurthle cell tumor*	*Lymphocytic thyroiditis*
Lesion	Solitary nodule	Diffuse
Hormonal status	Euthyroid	Hypothyroid
Antithyroid antibodies	Absent	Present
Colloid	Absent	Scanty
Hurthle cell	Abundant	Variable
Pleomorphism	Monomorphic	Pleomorphic
Nucleoli	Macronucleoli	Nucleoli present
Normal follicular cells	Scanty to absent	Present
Lymphocytes and plasma cells	Scanty to none	Present

TABLE 26.4: Medullary carcinoma versus Hurthle cell tumor

Feature	*Hurthle cell tumor*	*Medullary carcinoma*
Cytoplasmic granularity	Bluish granules	Reddish granules
Nucleoli	Large, prominent	Indistinct
Calcitonin	Negative	Positive
CK14	Positive	Negative

Immunocytochemistry: The Hurthle cells are positive for thyroglobulin, CK14, thyroid transcription factor (TTF), GLUT-4 and negative for calcitonin.

Diagnostic Difficulties

- Lymphocytic thyroiditis
- Hurthle cells in colloid goiter
- Medullary thyroid carcinoma: Both medullary thyroid carcinoma and Hurthle cell neoplasm show the presence of single cells and plasmacytoid appearance. Prominent nucleoli are the important distinguishing feature of Hurthle cell neoplasm.

Hyalinizing Trabecular Adenoma

Hyalinizing trabecular adenoma is a type of follicular cell neoplasm that exhibits trabecular arrangement of cells and prominent hyaline appearance. The hyaline is accumulated both intracellularly and extracellularly.

Cytology[39]

FNAC smears show cohesive clusters and dissociated tumor cells. The tumor cells are arranged radially in cohesive groups around hyaline material. The cells are round and monomorphic in appearance with abundant cytoplasm. The cytoplasm contains round paranuclear spherical body that stains purple-gray with MGG stain. Nuclei of the cells are single, moderately large, round, oval, polygonal, or spindled. The nucleocytoplasmic ratio is low. The intranuclear inclusions are seen frequently. Multiple intranuclear inclusions may cause nuclear enlargement and distortion. Intranuclear longitudinal grooves are also noted and may simulate papillary thyroid carcinoma (PTC).

Background of the smear shows abundant extracellular, dense, lumpy, and usually structure less material. The material stains pink with MGG and turquoise blue with Papanicolaou stain. This hyaline material may simulate amyloid material.

Differential Diagnosis

Papillary carcinoma of thyroid.

Papillary Carcinoma

Papillary thyroid carcinoma (PTC) is the most common thyroid tumor and represents about 60–80% of thyroid cancers. PTC is commonly noted in the age group of 30–50 years; however, no age is exempted from this tumor and this is the commonest malignancy of thyroid in the pediatric age. Females are more often affected than male and female: male ratio is 4:1. This tumor commonly spreads to the cervical lymph node. Occasionally, the patient first time presents with a palpable cervical lymph node only. The prognosis of PTC is excellent and overall 5 years survival rate is 80%.[40] The exact etiology of PTC is unknown. However, it has been noted that excess iodine intake in the diet increased the risk of PTC. External radiation of neck region also increases the chance of PTC. The incidence of PTC was 100-folds greater in the atom bomb survivors and after chernobyl accident of the atomic reactor in Ukraine.

Somatic rearranged intransformation/papillary thyroid carcinoma gene rearrangements (RET/PTC) occurs in 20–40% cases of PTC and creates a chimeric RET/PTC fusion oncogene. Here, entire RET tyrosine domain fuses with other genes to form this fusion gene.[41] Till now, a total of 17 chimeric fusion

genes have been identified with fusion of RET and other 12 genes. RET/PTC rearrangements can be demonstrated by reverse transcriptase-polymerase chain reaction (RT-PCR) or fluorescent in situ hybridization (FISH). The point mutation of BRAF gene is detected in 30–70% of PTC.[42] This particular mutation is associated with aggressive clinical behavior of PTC. Renin-angiotensin system (RAS) mutation is infrequent in PTC and is commonly noted in follicular neoplasm.[43]

Cytology (Figs 26.27 to 26.31)

- Arrangement: Papillary thyroid carcinoma shows multiple papillary structures, three-dimensional clusters and occasional discrete cells (**Box 26.14**). The papillae with central fibrovascular core are uncommonly seen[44] because the structures may be too big to come in the aspiration needle. The peripheral arrangement of cells in a papillary cluster is perpendicular to the inner layer of cells. Most of the time, the fragments or tip of the papillae is seen as three-dimensional clusters of cells. The nuclei of the cells show crowding and overlapping.
- Colloid: Colloid is scanty and round to oval dense hyperchromatic. In MGG smear, the colloid looks like dark magenta colored. At times, colloid is present as ropy streaks connecting clusters of cells. This is also mentioned as "bubble gum" colloid.[16]
- Cell cytoplasm: Individual cells in PTC are oval with moderate amount of cytoplasm and well-defined cell border. The cytoplasm of the cell is often dense granular and appears like squamous cell. Cytoplasm with fine granularity resembles oncocytes. In case of cystic PTC, there may be significant clearing of the cytoplasm giving rise to clear cell appearance. These cells may be confused with histiocytes in cystic goiter.
- Nucleus: Nuclei of the cells in PTC are enlarged and mild pleomorphic. The nuclei show pale chromatin and small

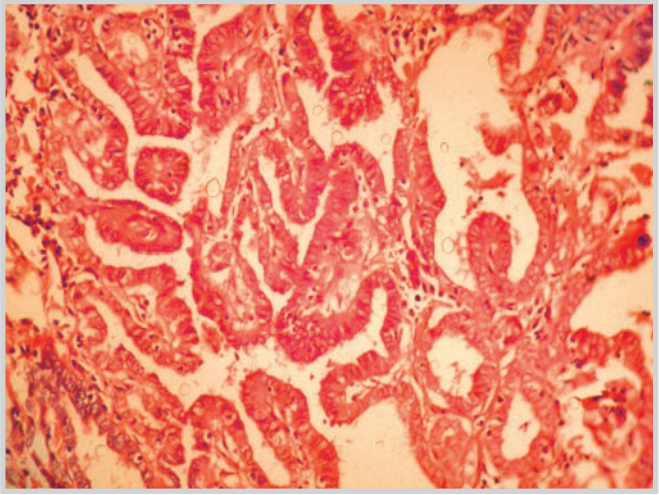


Fig. 26.27A: Papillary carcinoma of thyroid: Histopathology section shows multiple papillae with fibrovascular core (H & E × MP)

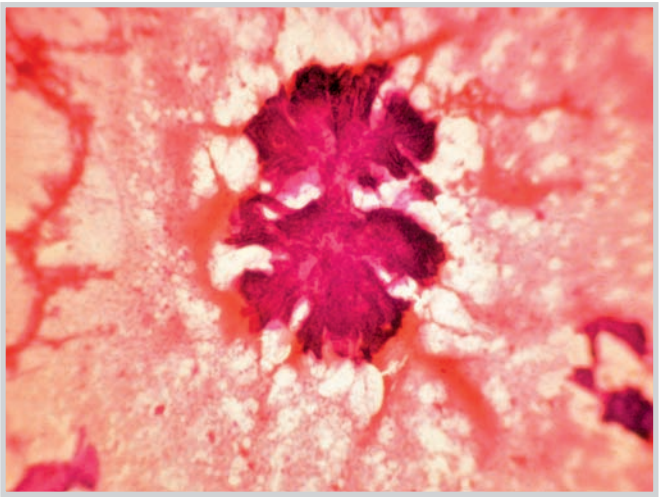


Fig. 26.28: Papillary carcinoma of thyroid: Papillary cluster of cells (H & E × MP)

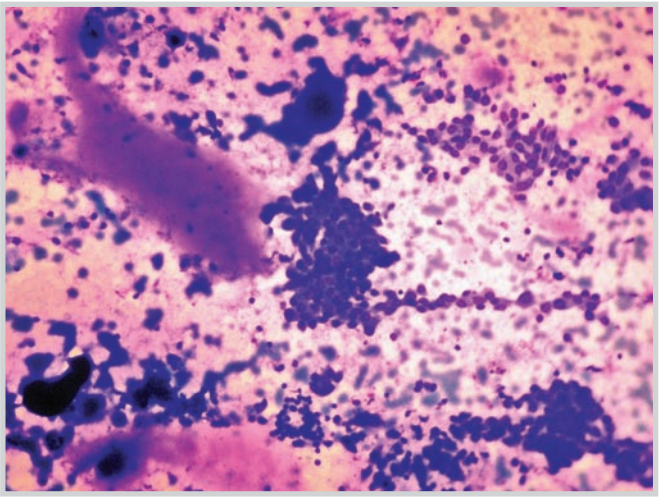


Fig. 26.27B: Papillary carcinoma of thyroid: Clusters and dissociated cells in the background of chewing gum-like thick colloid (MGG × MP)

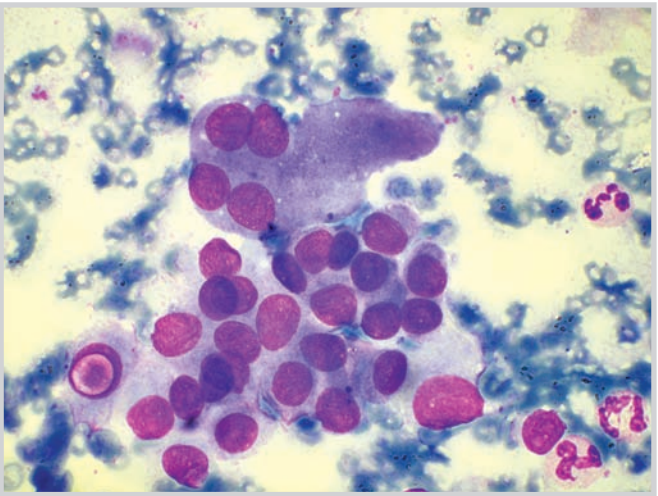


Fig. 26.29: Papillary carcinoma of thyroid: Prominent intranuclear pesudoinclusion (MGG × OI)

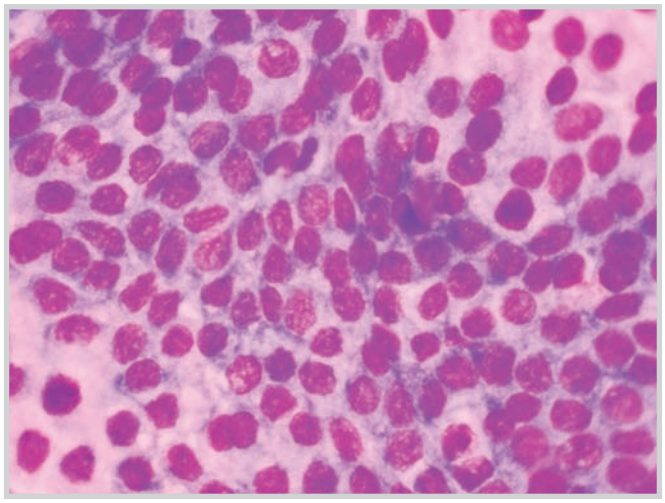


Fig. 26.30A: Nuclear groove in papillary carcinoma of thyroid (MGG × OI)

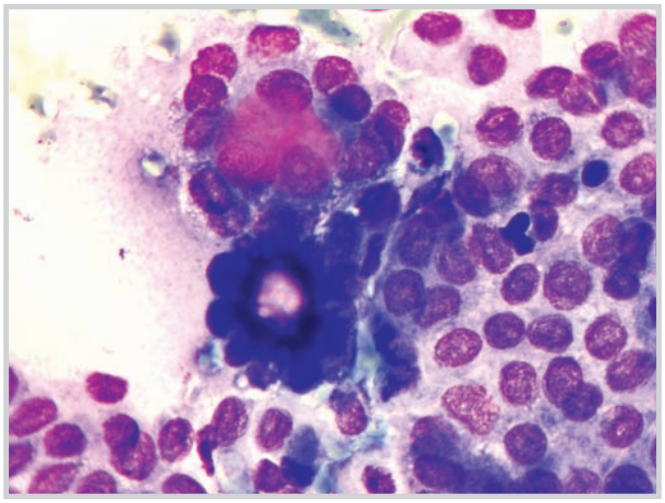
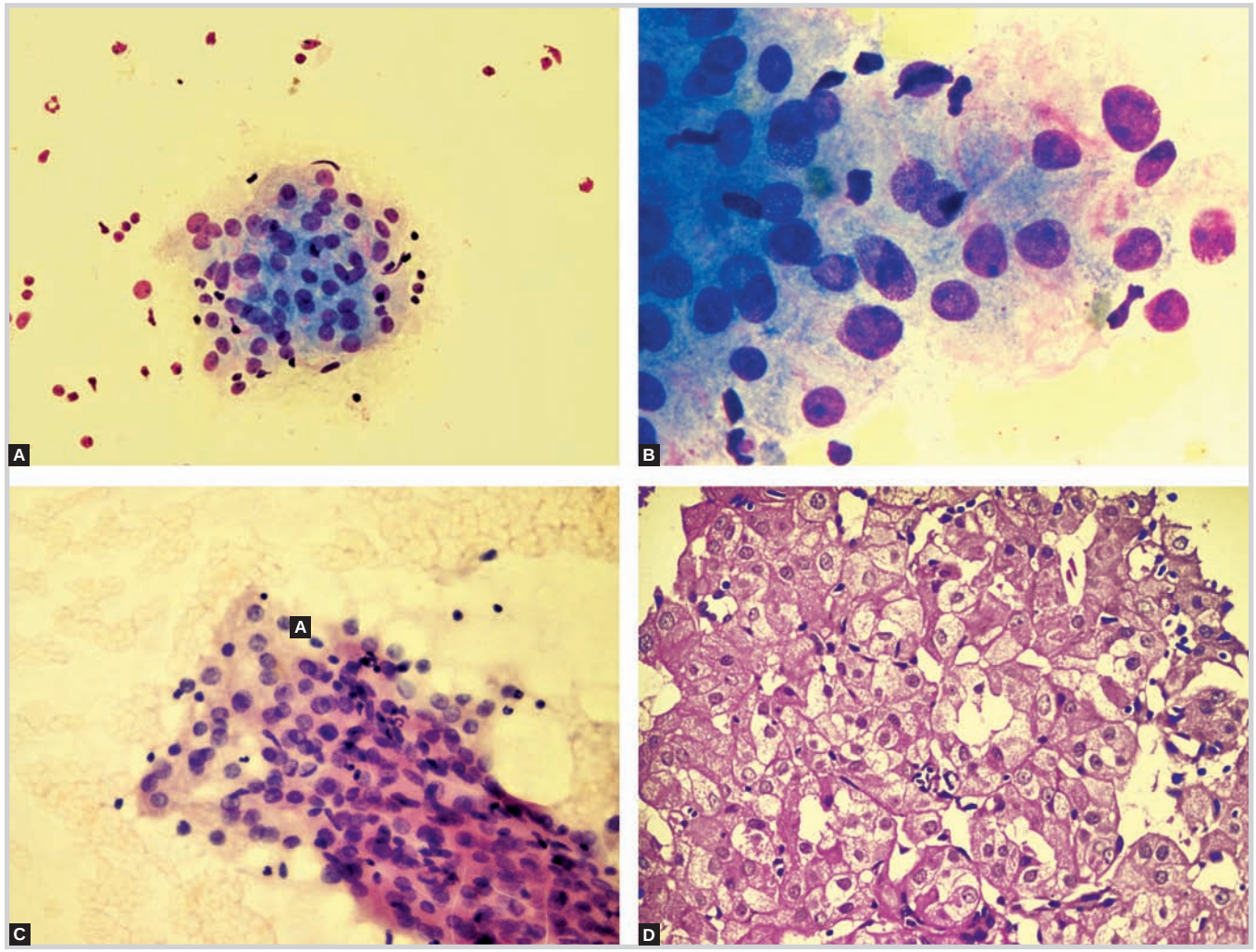


Fig. 26.30B: Concentric lamellated psammoma body in papillary carcinoma of thyroid (MGG × HP)



Figs 26.31A to D: Clear cell changes in papillary carcinoma of thyroid: (A) Small cluster of cells with clear cell changes. (MGG × MP), (B) Higher magnification showing better morphology of the clear cells. (MGG × OI), (C) Papillary cluster of cells. (H & E × MP), (D) Cell block preparation of the same (H & E × MP)

BOX 26.14 Papillary carcinoma

- Papillary arrangement
- Intranuclear grooves
- Intranuclear pseudoinclusions
- Optically clear nucleus
- Thick chewing gum-like ropy colloid
- Psammoma bodies
- Multinucleated giant cells
- Histiocytes and lymphocytes

BOX 25.15 Psammoma body

Appearance: Round, concentric, lamellated and calcified structures
Pathogenesis:
- Dystrophic calcification
- Thrombosis and focal necrosis of tips of papillae followed by calcification

Incidence in papillary thyroid carcinoma (PTC): 10–35% cases
Other thyroid lesions with PB: Nodular goiter and Hashimoto's thyroiditis
Differential diagnosis:
- Thick colloid
- Calcified material

PBs noted in Diseases other than thyroid:
- Meningioma
- Papillary cystic adenocarcinoma of ovary
- Chromophobe renal cell carcinoma
- Psammoma carcinoma of the peritoneum
- Cholangiocarcinoma

eccentric nucleoli. The characteristics nuclear morphology of PTC is:

– Optically clear nucleus: The nucleus of PTC shows pale, fine and powdery chromatin. This type of nucleus in alcohol fixed smear gives the so called "Orphan Annie eye" or optically clear appearance. Exact molecular etiology of the optically clear nucleus is not known. Probably RET oncogene expression is responsible for this change.
– Intranuclear groove: It is due to the deep longitudinal folding of the nuclear membrane parallel to the long axis of the nuclei. This is well-appreciated in Papanicolaou's stained smear. Nuclear groove is a nonspecific finding but it is a useful diagnostic feature when detected in an enlarged nucleus with pale chromatin
– Intranuclear pesudoinclusion: The intranuclear pseudoinclusions represent the cytoplasmic invaginations within the nucleus. They are also seen in more than 90% of PTC. Intranuclear inclusions are best seen in Papanicolaou's or hematoxylin and eosin (H & E) stained smear. The major characteristics of intranuclear pesudoinclusion are:
  a. Large membrane bound structure
  b. Condensed outer rim of chromatin
  c. Clear than the surrounding chromatin
  d. Identical tinctorial color of the cytoplasm

The other structures which can be mistaken as intranuclear pseudoinclusions are: (1) nucleoli and (2) overlapping red blood cell (RBC) on the nucleus.[45] Intranuclear inclusions are not specific for PTC and can be seen in other neoplastic and non-neoplastic conditions of thyroid such as hyalinizing trabecular adenoma, MC, Hurthle cell tumor, LT and nodular goiter.

- Giant cells: Multinucleated giant cells are frequently noted in PTC. These cells are huge with homogenous cytoplasm and contain multiple nuclei. Multinucleated giant cells in PTC are positive for CD68 immunostaining. These are histiocytes and not tumor cell.[46]
- Psammoma bodies (**Box 26.15**): Psammoma bodies (PBs) are round, concentric, lamellated, and calcified structures. In cytology smear, they are 50–70μ size, round-shaped, glassy in look and takes dark blue to black color in MGG staining and brown to black color in Papanicloalou's stained smear.
- There are many theories of pathogenesis of PB formation. It was considered that in the process of dystrophic calcification, the necrotic tumor cells act as seed around which minerals are deposited in a concentric manner.[47] LiVolsi et al. suggested that true PBs are formed due to focal areas of infraction in tips of the papillae because of thrombosis of the small vessels. However, these are different from dystrophic calcification as they are irregular or jagged and rarely show lamellations.[48] Johannesen and Sobrinho-Simoes et al.[49] did detailed ultrastructural study of thyroid PBs and suggested that PBs represent two end stage biologic processes: (1) thickening of the basal lamina of the small vessels leading to thrombosis, calcification and necrosis, (2) necrosis and calcification of the intralymphatic tumor thrombi in adjacent nontumor thyroid areas.
- The presence of PBs in the aspirate is pathognomonic for PTC. FNAC smears of 11–35% cases of PTC show PB.[50] PBs are particularly helpful in the diagnosis of cystic papillary carcinoma. It has been recommended that the presence of PBs in FNAC of solitary thyroid nodule should have surgical excision of the thyroid due to high risk of malignancy.[51]
- Psammoma bodies should be distinguished from the inspissated colloid and calcified material due to degenerative changes in thyroid. It is important to note that PBs are also found in nodular goiter and LT.[52]
- Macrophages and lymphocytes: These cells are also noted in the aspirates of PTC in variable amount. Macrophages are usually abundant in PTC with cystic changes. Lymphocytes and occasional plasma cells may also be seen in PTC and their presence may lead to the false negative diagnosis of LT.

A combination of cytological features is needed to diagnose a case of PTC. The nuclear features are the most important among all the other diagnostic criteria of PTC. In the NCI sponsored symposium on cytology, in 2007 some major diagnostic criteria of PTC have been recommended as:[24]

The major diagnostic criteria of PTC
- Enlarged, oval "irregular" nucleus
- Eccentric and often multiple micronucleoli
- Fine, pale chromatin
- Longitudinal nuclear grooves
- Intranuclear pseudoinclusions.

Similarly the minor diagnostic criteria of PTC criteria include:

- Papillary cytoarchitecture
- Syncytial monolayers
- Squamoid cytoplasm
- Psammoma bodies
- Multinucleated giant cells
- Histiocytoid cells and cellular swirls.

- Variants of PCT: There are several variants of PCT which are often encountered in routine reporting. These cases are difficult to diagnose on cytology alone. The salient cytological features of these variants are described below:[53-58]
- Follicular variant of papillary carcinoma of thyroid[54,55] (**Fig. 26.32** and **26.33**): This is one of the common variants of PTC. Typical papillary arrangement of the cells is missing in this variety and the smears show large number of microfollicles. The exact diagnosis largely depends on the identification of the nuclear features. Cytology smears of FVPCT are often misdiagnosed as follicular neoplasia and this tumor is the commonest cause of false negative diagnosis of PTC. Characteristically, the aspirate shows multiple follicles and also syncytial-like cells (**Box 26.16**). Individual cells usually show nuclear features of PTC which is very important to recognize. Chewing gum like thick colloid is seen in the background. Rarely multinucleated giant cells and PB are noted.
- Cystic papillary carcinoma: It is not in fact a true variant of PTC. PTC often undergoes extensive cystic changes and aspirate yields fluid. The smears show occasional papillae. Large number of foamy histiocytes is seen along with loose clusters and discrete tumor cells. The individual cells show characteristic nuclear features of PTC. Cystic PTC is one of the common sources of false negative diagnosis.
- Tall cell variant[53] (**Figs 26.34A and B**): This is an aggressive variant of PTC with poor clinical outcome. The tumor commonly affects older patient. The cytology smear shows large numbers of tall columnar looking cells with abundant eosinophilic cytoplasm resembling oncocytic cells. The cell in tall cell variant should be three times tall as they are wide. Nuclei of the cells are basally placed and show features of papillary carcinoma of thyroid (**Box 26.17**). Moderate amount of lymphocytes may be seen in the background of the smear. Tall cell variant of PTC is difficult to recognize on the cytology smears.
- Columnar cell variant: In case of columnar cell variant, the smears show crowded, stratified clusters of elongated cells with cigar shaped nuclei. The cells are tall columnar with scanty cytoplasm. The nuclei are cigar shaped, hyperchromatic,

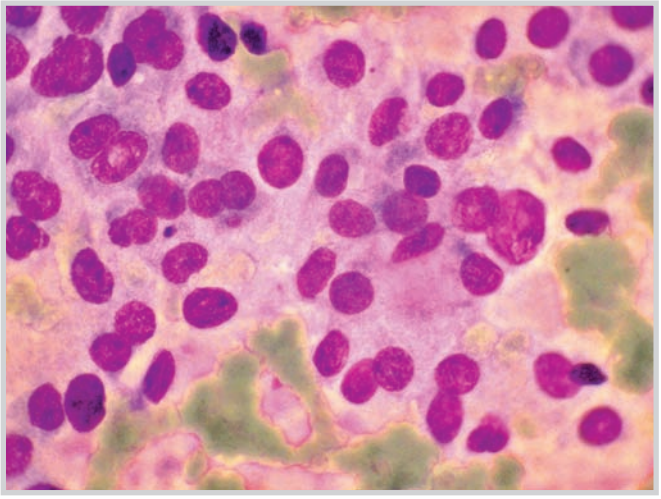


Fig. 26.33B: Higher magnification showing intranuclear inclusion and grooving in FVPCT (MGG × OI)

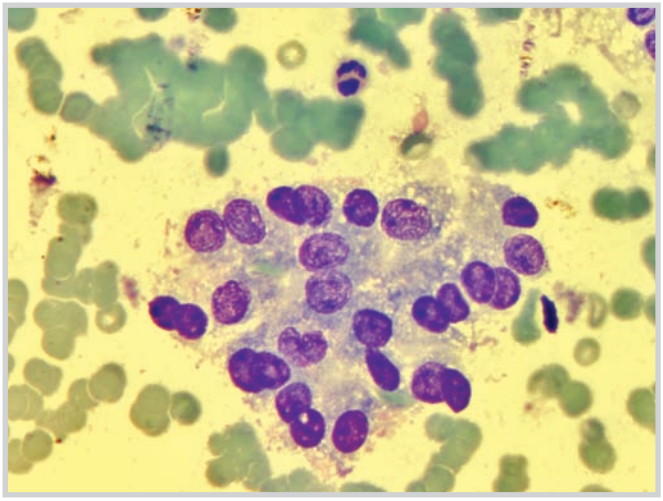


Fig. 26.32: Follicular variant of papillary carcinoma of thyroid (FVPCT): follicles lined by cells with nuclear groove (MGG × HP)

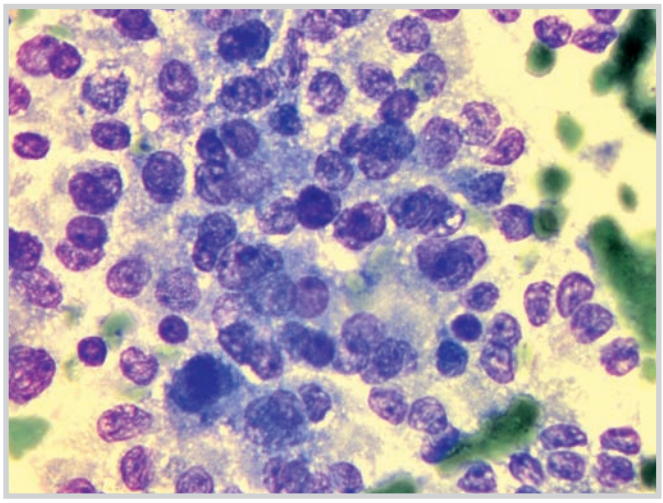


Fig. 26.33A: Microfollicles and discrete follicular cells with nuclear grooving in FVPCT (MGG × HP)

BOX 26.16 Follicular variant of papillary carcinoma of thyroid

- Large number of microfollicles
- Nuclear features of papillary thyroid carcinoma (PTC)
- Chewing gum like thick colloid
- Rarely multinucleated giant cells and psammoma body

Differential diagnosis: Follicular neoplasia

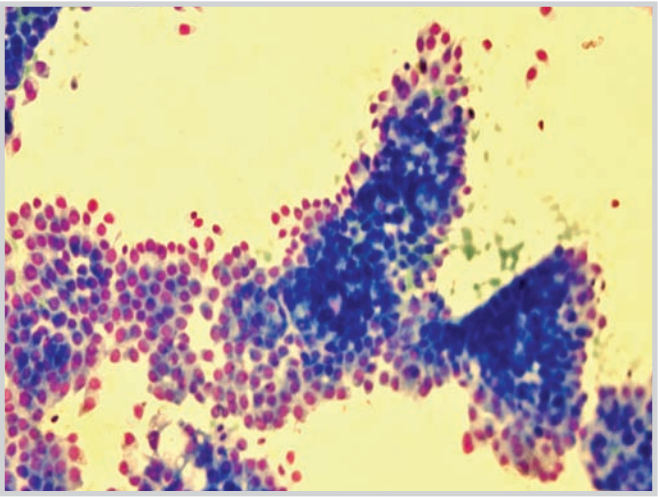


Fig. 26.34A: Tall cell variant of papillary carcinoma of thyroid: Papillary clusters of tall cells (MGG × MP)

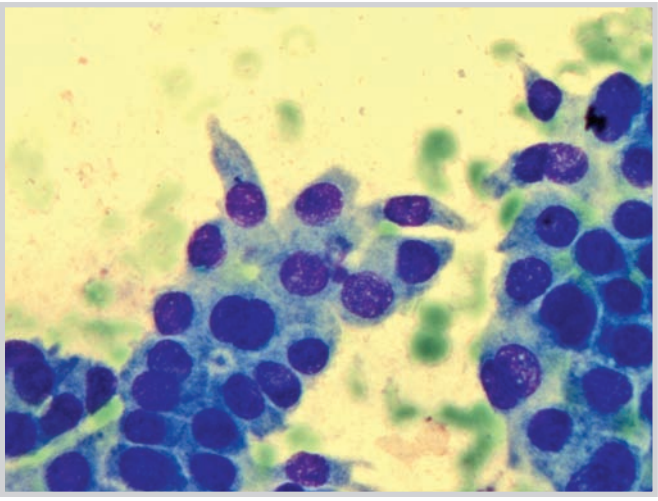


Fig. 26.34B: Tall cell variant of papillary carcinoma of thyroid: Higher magnification showing tall cell (MGG × OI)

BOX 26.17 Tall cell variant

- Large numbers of tall columnar cells
- Abundant eosinophilic cytoplasm
- Basally placed nuclei
- Lymphocytes
- Nuclear features of papillary thyroid carcinoma (PTC)

monomorphic in appearance with indistinct nucleoli. Unlike tall cell variant, these cells are taller; cytoplasm is scanty and lack classical nuclear features of PTC.

- Diffuse sclerosing variants:[58] This is a relatively uncommon variant of PTC and usually occurs in younger patients. PTC is noted within the dense fibrous stroma. The smears characteristically show syncytial clusters of tumor cells in the background of scattered lymphocytes (**Box 26.18**). Squamous metaplastic cells and abundant PBs are also noted.

BOX 26.18 Diffuse sclerosing variants

- Syncytial clusters of cells having typical nuclear features of papillary thyroid carcinoma (PTC)
- Abundant psammoma bodies
- Metaplastic squamous cells
- Dense lymphocytic infiltrate.

BOX 26.19 Oncocytic variant

- Cell in clusters, papillae and discrete
- Abundant eosinophilic granular cytoplasm
- Nuclear features of papillary thyroid carcinoma (PTC)

- Oncocytic variant: This is also a relatively uncommon variant of PTC. FNAC smears show abundant oncocyte like cells in clusters, follicular and papillary arrangement (**Box 26.19**). The individual cells show abundant eosinophilic granular eosinophilic cytoplasm caused by mitochondrial proliferation. The nuclei of the cells show features of PTC. This variant comes in differential diagnosis of Hurthle cell tumor and MC.
- Warthin like PTC: In this tumor, the cytology smear shows papillary clusters of oncocyte-like cells along with discrete lymphocytes and plasma cells. The tumor cells show classical nuclear features of malignancy. The FNAC smears of this entity may often be misdiagnosed as LT. However, the nuclear features of PTC are helpful in diagnosis.

Differential Diagnosis

- Chronic lymphocytic thyroiditis
- Hyalinizing trabecular adenoma: The frequent nuclear grooves and intranuclear pseudoinclusion in hyalinizing trabecular adenoma may be misinterpreted as PTC.

Variants of Papillary Carcinomas: Differential Diagnosis

- Follicular neoplasm versus FVPCT
- Lymphocytic thyroiditis versus diffuse sclerosing variants of PTC.

Medullary Carcinoma

Medullary carcinoma represents almost 10% of thyroid carcinomas. The tumor is composed of parafollicular or C cell of thyroid and shows neuroendocrine morphology. The majority of the MC is sporadic and do not show any association with age or sex. Approximately, one-fourth of MC is inherited and is related with multiple endocrine neoplasia (MEN) syndrome 2A and 2B. Majority of MC (90%) secretes calcitonin hormone and therefore

serum estimation of calcitonin may be used as a screening test of MC. The tumor metastasizes to the regional cervical lymph node and occasionally the patient first time presents with an enlarged lymph node. Therefore, MC may come in the differential diagnosis of positive lymph nodal mass with unknown primary.

Cytology[59-62] (Figs 26.35 to 26.41)

Aspirates of MC show predominantly dissociated cell population (**Box 26.20**). Occasional loose syncytial aggregates or follicular pattern may be present. Three types of cells are present:

1. Round plasmacytoid cells
2. Spindle cells
3. Polygonal cells

The round plasmacytoid cells are commonly seen. The cells have monomorphic round central to eccentric nuclei. Cytoplasmic border is well-defined and there are reddish granules in the cytoplasm. The nuclei have stippled chromatin giving salt and pepper appearance like that of neuroendocrine tumor. Polygonal angulated cells have well-defined reddish cytoplasm.

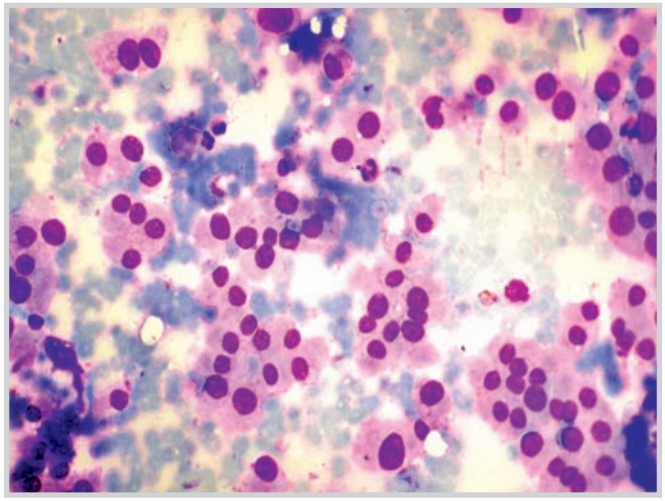


Fig. 26.35: Discrete cells in medullary carcinoma of thyroid (MGG × MP)

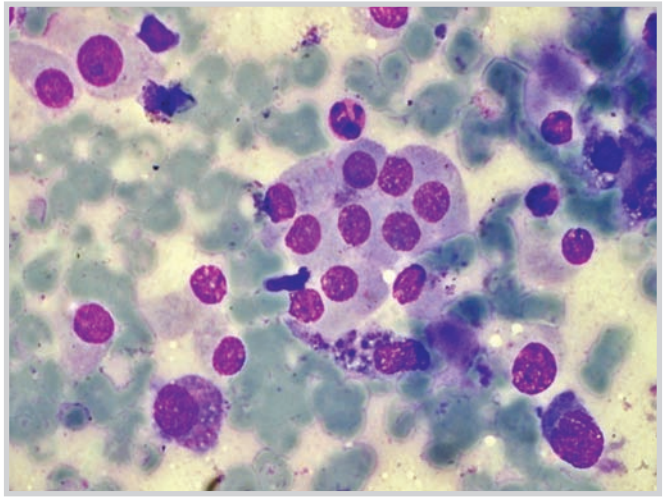


Fig. 26.36A: Round cells with moderate amount of cytoplasm having eccentric nuclei in medullary carcinoma of thyroid (MGG × MP)

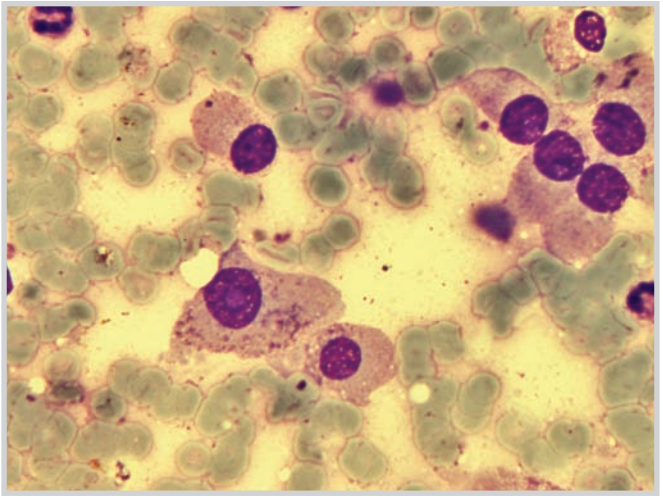


Fig. 26.36B: Medullary carcinoma of thyroid: Large polygonal cells (MGG × HP)

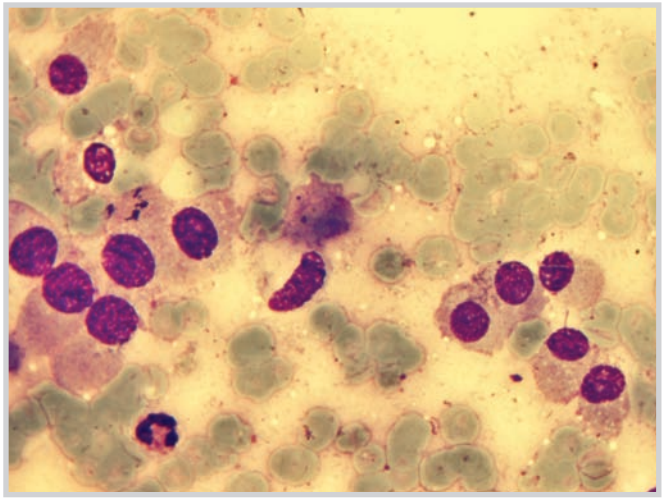


Fig. 26.36C: Medullary carcinoma of thyroid: Occasional spindle-shaped cell (MGG × HP)

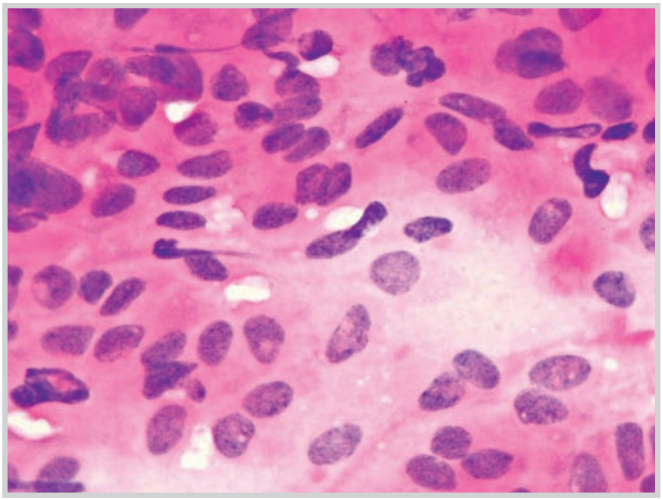


Fig. 26.36D: Medullary carcinoma of thyroid: Many spindle cells (H & E × MP)

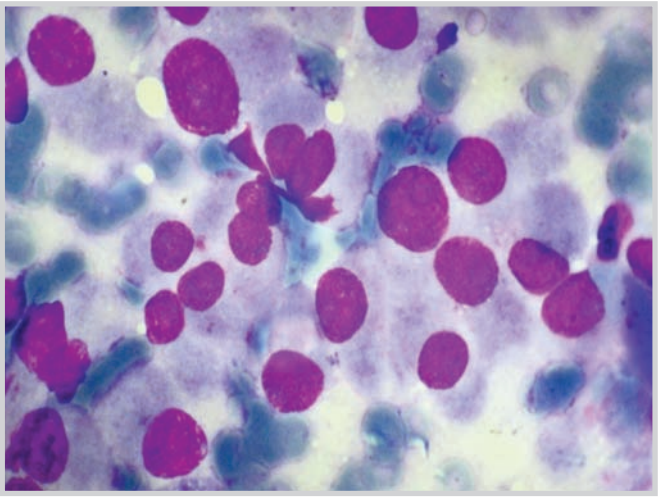


Fig. 26.37: Higher magnification showing cells with stippled nuclear chromatin in medullary carcinoma of thyroid (MGG × OI)

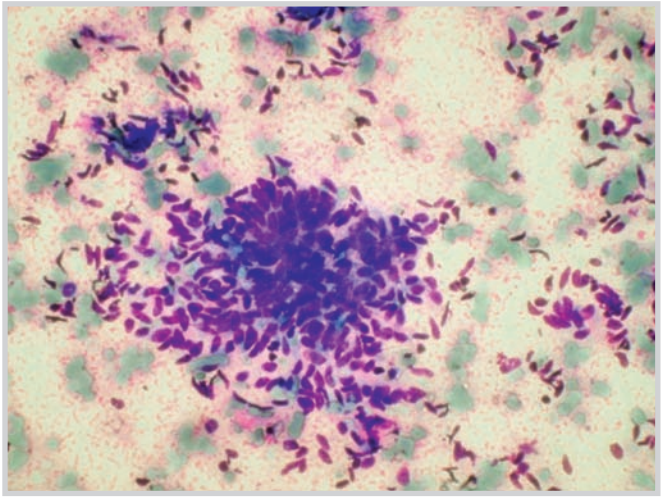


Fig. 26.40: Predominantly spindle cells in medullary carcinoma of thyroid (MGG × HP)

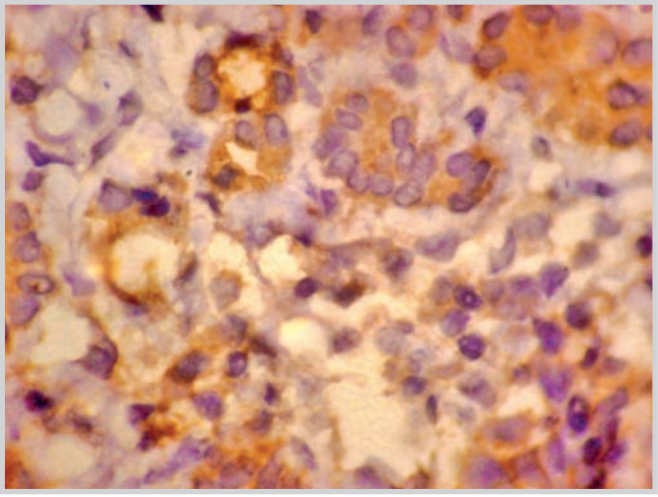


Fig. 26.38: Calcitonin positivity in medullary carcinoma of thyroid (Immunostain × HP)

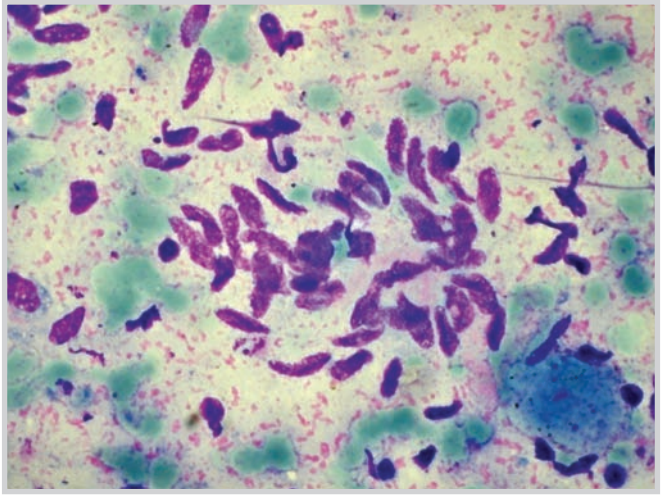


Fig. 26.41: Loose cluster of spindle cells with elongated blunt ended nuclei in medullary carcinoma of thyroid (MGG × HP)

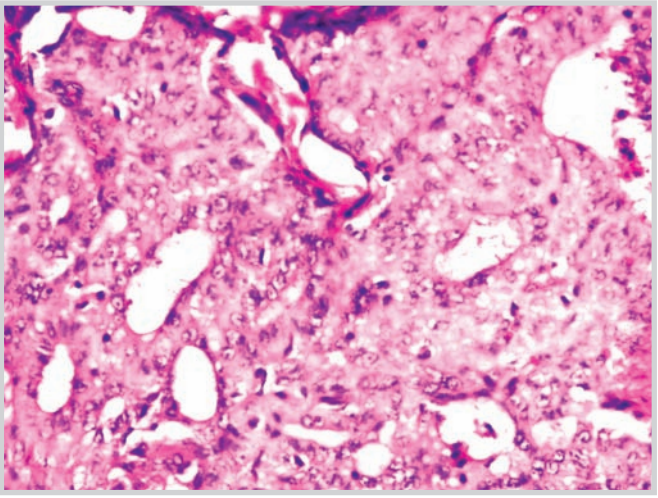


Fig. 26.39: Histopathology section of medullary carcinoma of thyroid (H & E × HP)

BOX 26.20 Cytologic features of medullary carcinoma

- Predominantly dissociated cells
- Cells:
  - Round plasmacytoid cells
  - Spindle cells
  - Polygonal cells:
    - Reddish granular cytoplasm
    - Salt and pepper chromatin
    - Amorphous acellular pinkish amyloid material
    - Squamoid cells, small cells, clear cells
    - Immunocytochemistry: Positive for calcitonin, chromogranin and synaptophysin.

These cells resemble oncocytes. The third cell types are spindle shaped cells with elongated cigar shaped nuclei. The presence of predominant spindle cells may create diagnostic difficulty with fibroblastic tumor. These cells show less nuclear pleomorphism and rarely may have small nucleoli. In addition, uncommonly squamoid cells, small cells and clear cells may also be seen. Majority of times, mixed population of cells or plasmacytoid cells are present. Occasional intranuclear pseudoinclusion and multinucleated giant cells in MC may be noted.

Pale blue amorphous and acellular amyloid material is seen in 80% of MC cases. Amyloid is round or string like with smooth edged and fibrillar in appearance. This amyloid is present discretely in the smear or may be surrounded by tumor cells. At times, it is difficult to differentiate amyloid from colloid on routine MGG smear. However, amyloid is round with smooth demarcated margin and fibrillar in contrast to cracking artifact and sharp angulated edges of colloid. Special stain such as Congo red stain may be needed to confirm the presence of amyloid. Amyloid shows apple green birefringence in polarized light when stained by alkaline Congo red.

Immunocytochemistry

- Calcitonin positivity is specific of MC
- Cells of MC are also positive for neuroendocrine markers such as chromogranin, neuron-specific enolase (NSE) and synaptophysin.
- Positive for carcinoembryonic antigen (CEA)
- Negative for thyroglobulin.

Differential Diagnosis

- Hurthle cell neoplasm
- Papillary carcinoma of thyroid
- Follicular neoplasm
- Metastatic tumors such as malignant melanoma, plasmacytoma, neuroendocrine carcinoma.

Anaplastic Carcinoma

This is a highly aggressive malignant tumor of thyroid and represents less than 5% of thyroid carcinoma.[32] This is commonly found in the elderly patient (average age 65 years) and is very uncommon under the age of 50 years. In majority of the cases, the patient gives a history of pre-existing colloid goiter, FCa or PTC.

Cytology (Figs 26.42 to 26.44)

On histology, anaplastic carcinoma is of two types. The first type is totally undifferentiated without any follicular, trabecular or nest. However, the epithelial nature of this tumor is evident. The tumor shows squamoid differentiation and keratinization. In other type, the tumor gives sarcomatoid appearance and is composed of spindle cells and giant cells. There are large areas of polymorphonuclear leucocytic infiltration in the tumor. Osteoid, cartilaginous and skeletal muscle differentiation is seen.

Cytology picture of anaplastic carcinoma mainly depends on its histological subtype.

Anaplastic carcinoma of thyroid shows necrosis along with loose clusters and discrete malignant cells and polymorphonuclear leucocytes (**Box 26.21**). The tumor cells are large with markedly pleomorphic bizarre nuclei having irregular nuclear margin and single to multiple nucleoli. Many multinucleated cells are seen.

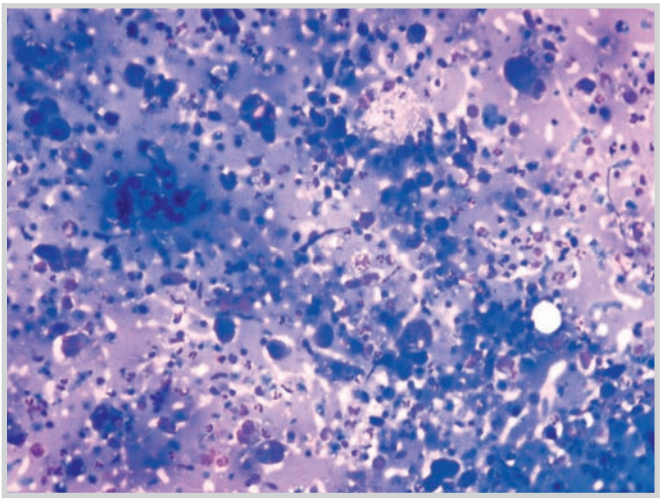


Fig. 26.42: Many discrete malignant cells in a necrotic background in anaplastic carcinoma of thyroid (MGG × MP)

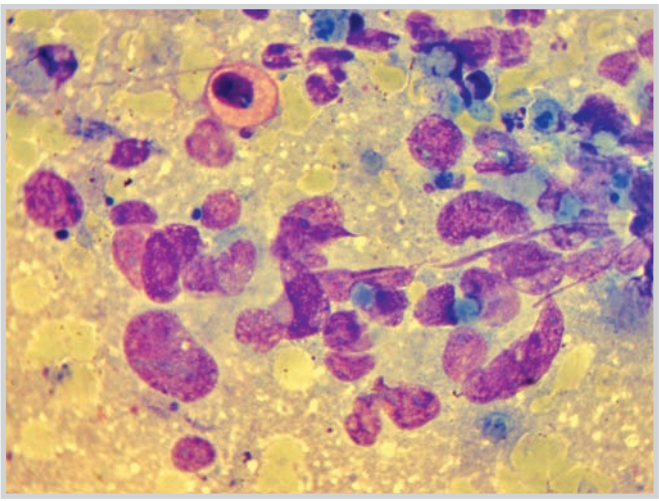


Fig. 26.43: Malignant cells have enlarged moderately pleomorphic nuclei in anaplastic carcinoma of thyroid (MGG × HP)

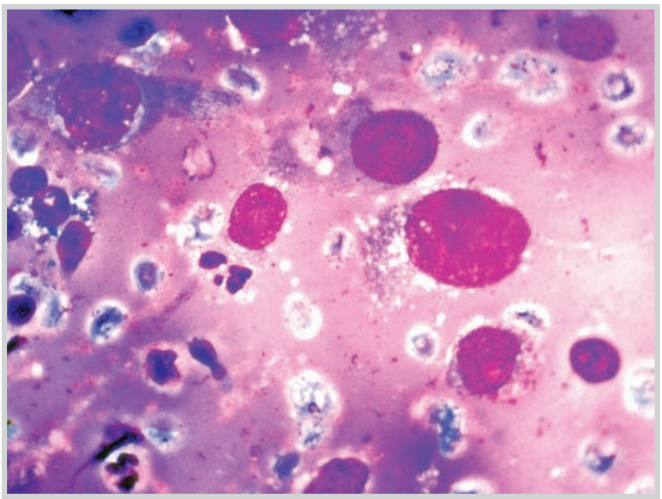


Fig. 26.44: Higher magnification showing better cell morphology in anaplastic carcinoma of thyroid. The cells have markedly enlarged nuclei with multiple prominent nucleoli (MGG × OI)

BOX 26.21 Cytological features of anaplastic carcinoma

- Necrosis
- Polymorphs
- Discrete large pleomorphic tumor cell
- Prominent nucleoli, irregular membrane
- Neutrophil cannibalism
- Spindle cells
- Squamoid cells
- Multinucleated giant cells

The tumor cells may show neutrophilic cannibalism. Occasionally, the smear may show predominant spindle cell population with moderate pleomorphism. Osteoclast like giant cells, chondroid and osseous material may also be noted.[63] Marked background necrosis and leucocytes infiltration may obscure the malignant cells and the tumor may rarely be missed as acute thyroiditis. If there is any suspicion of underlying tumor, then a repeat FNAC is mandatory. Predominant population of spindle cells in anaplastic carcinoma may pose diagnostic difficulty with MC, fibrosarcoma or hemangioendothelioma. The associated large bizarre cells and background necrosis may be helpful in the diagnosis of anaplastic carcinoma. In difficult situation, immunocytochemistry in the cell block preparation is always helpful.

Immunocytochemistry

The cells are negative for thyroglobulin and positive for vimentin.

Differential Diagnosis

- Acute thyroiditis
- Spindle cell variant of MC
- Other sarcomas: Fibrosarcoma, malignant hemangioendothelioma, etc.
- Metastatic giant cell tumors from lung.

Insular Carcinoma

Insular carcinoma (IC) is a poorly differentiated carcinoma and represents 4–7% of all thyroid carcinoma.[64] The cell of origin of IC is follicular cells of thyroid. It commonly occurs in elderly patient. The biological behavior of IC is intermediate between well-differentiated carcinoma and undifferentiated carcinoma. Distant metastasis of this tumor is common at the time of first presentation. The 5 years survival rate is 50%.

Cytology (Figs 26.45 to 26.49)

On histology, IC shows insular pattern of growth and solid to microfollicular arrangement. In insular type of growth, the small round tumor cells are arranged as nests surrounded by thin fibrovascular sptae. The tumor cell may also be arranged in solid sheets to varying amount of small microfollicles.

Cytology smears of IC shows predominantly discrete cells. Depending on histological type variable amount of microfollicles and clusters of cells are seen. The tumor cells are round to oval with scanty to moderate cytoplasm and often give rise to plasmacytoid appearance (**Box 26.22**).[65-67] The nuclei are monomorphic, round with regular nuclear margin and inconspicuous nucleoli. Nuclear

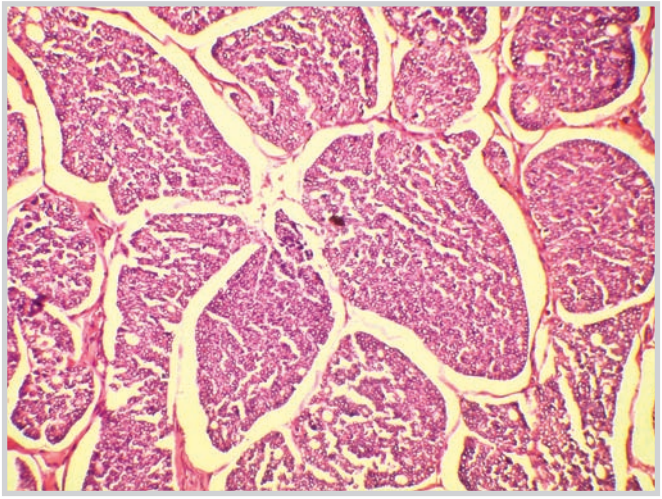


Fig. 26.45: Histopathology of insular carcinoma of thyroid showing large nests of cells separated by fibrous septae (H & E × MP)

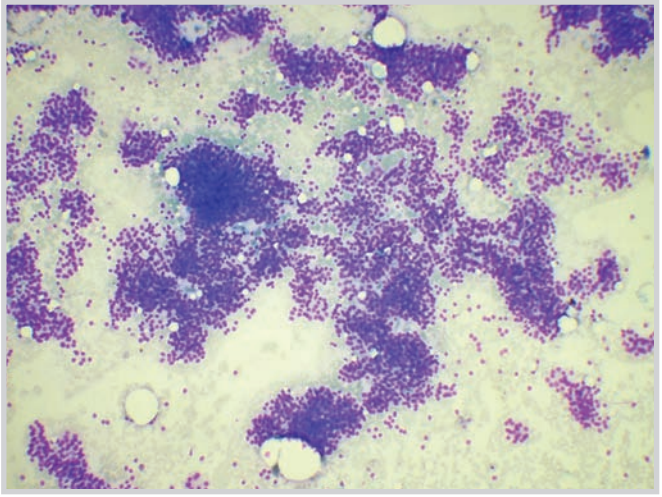


Fig. 26.46: Abundant cells in sheets and discretely placed in insular carcinoma of thyroid (MGG × LP)

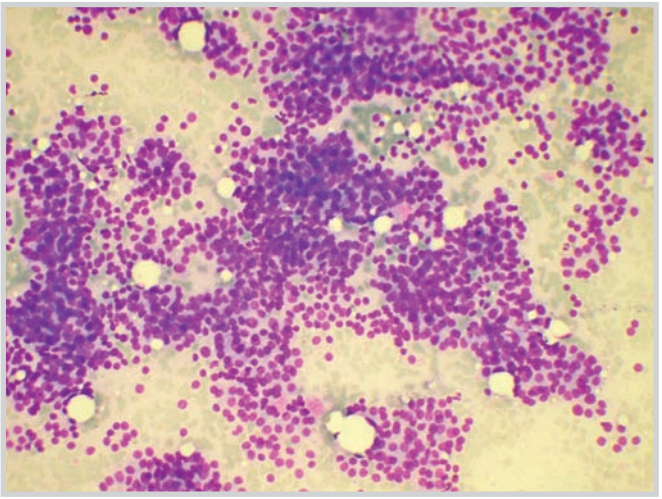


Fig. 26.47: Mainly solid sheets and occasional microfollicles in insular carcinoma of thyroid (MGG × MP)

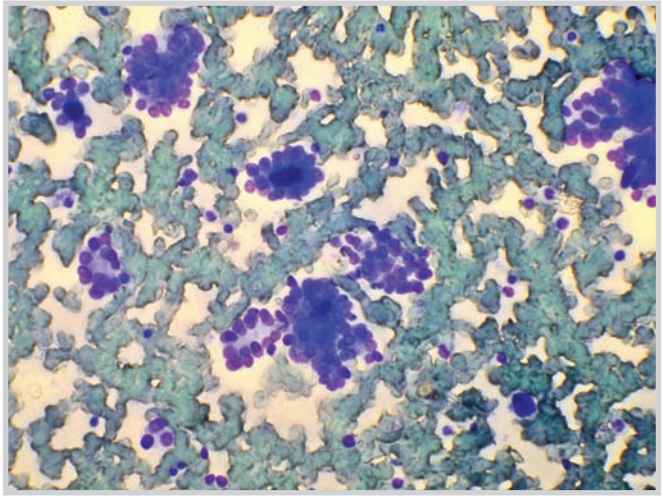


Fig. 26.48: Many microfollicles with thick colloid inside them in in insular carcinoma of thyroid (MGG × MP)

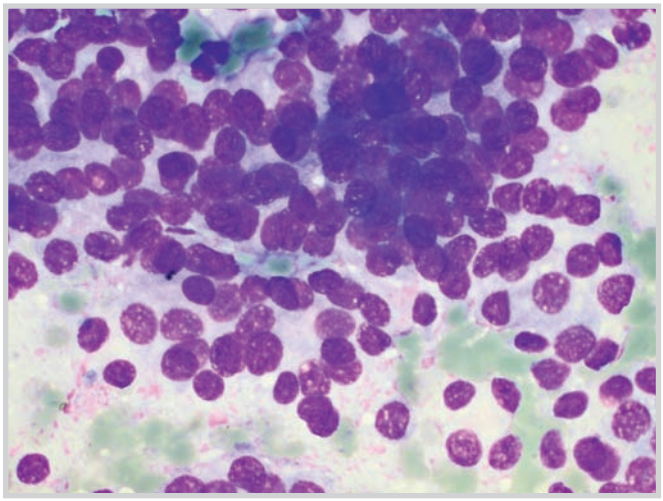


Fig. 26.49: Higher magnification showing round monomorphic cells with occasional nuclear grooves (MGG × HP)

BOX 26.22 Cytology of insular carcinoma

- Dissociated cells
- Occasional microfollicles
- Small round cells
- Monomorphic round nuclei and inconspicuous nucleoli

grooves and intranuclear pseudoinclusions are also noted and may simulate PTC. On cytology smear, it is difficult to diagnose IC with confidence. Microfollicular pattern present in IC may simulate FN.

Immunocytochemistry

Insular carcinoma is positive for thyroglobulin and thyroid transcription factor 1 (TTF-1); and negative for calcitonin.

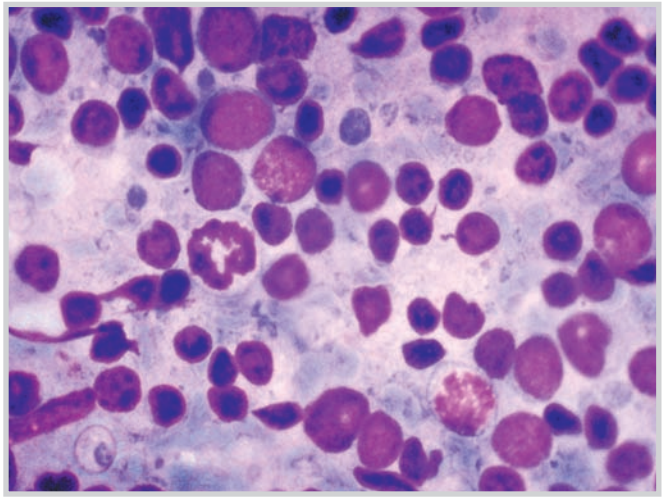


Fig. 26.50: Large discrete cells in diffuse large B-cell lymphoma of thyroid (MGG × HP)

Differential Diagnosis

- Papillary thyroid carcinoma
- Follicular neoplasm

Lymphomas[68-71]

Primary thyroid lymphoma, labeled as mucosa-associated lymphoid tissue lymphoma (MALTOMA), represents less than 5% of all thyroid malignancies and constitutes less than 2% of all extra-nodal lymphomas. As thyroid involvement is common in any systemic NHL, so this possibility should always be excluded before the diagnosis of primary NHL. Primary thyroid NHL usually occurs in elderly patient with a female predilection. Hashimoto's thyroiditis is a risk factor of NHL of thyroid.

Cytology

The morphology of thyroid NHL is essentially same as that of NHL in other body sites. The most of the cases of thyroid lymphomas are B cell type. Three histological subtypes of B NHL are seen:

- Diffuse large B-cell lymphoma (DLBCL)
- Marginal zone B-cell lymphoma (MZBCL)
- Mixed MZBCL and DLBCL type

Diffuse large B-cell lymphoma: Cytology smear show discrete large lymphoid cells with scanty to moderate amount of basophilic cytoplasm. The nuclei show coarse chromatin pattern with prominent nucleoli (**Fig. 26.50**).

Marginal zone B-cell lymphoma: FNAC of MZBCL shows lymphoid cells that are twice the size of small lymphocytes. Cells show scanty basophilic cytoplasm, irregular nuclear margin with small inconspicuous nucleoli (**Figs 26.51 and 26.52**).[70] Variable amounts of normal thyroid follicular cells may be admixed with the lymphoid cells. Florid lymphoid cell population in LT may be difficult to distinguish from MALTOMA. At times, LT may coexist with thyroid lymphoma. Immunocytochemistry or flow cytometry may be needed to establish the monoclonality of lymphoma to resolve the diagnostic confusion.

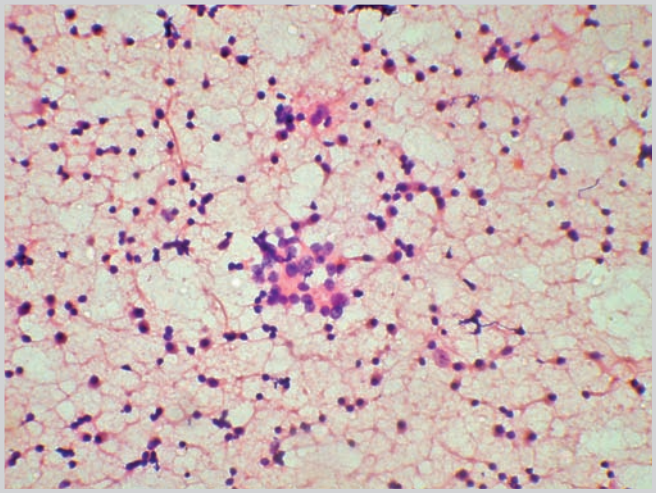


Fig. 26.51: Many lymphoplasmacytoid cells along with benign thyroid follicular cells in MALTOMA of thyroid (H & E × MP)

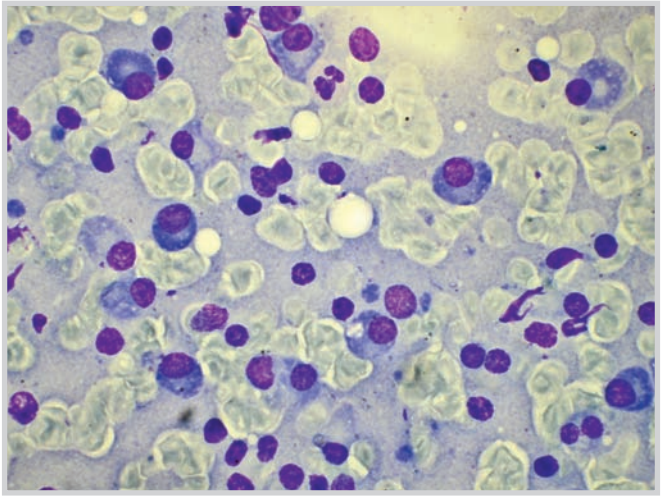


Fig. 26.52: Higher magnification showing immature lymphoid cells and plasmacytoid cells with eccentric nuclei in MALTOMA of thyroid (MGG × OI)

Differential Diagnosis
Chronic LT.

Metastatic Malignant tumors

Metastatic malignant tumor of thyroid represents about 0.1% of all thyroid cancer.[72] However, the incidence is reported more in autopsy cases (about 25%).[73] The majority of the cases of thyroid metastasis are due to direct extension of the malignancy from the adjacent structures such as larynx or esophagus. The other distant common sources of the primary tumors are kidney, lung and breast.[74-76] Metastatic tumors in thyroid is suspected if (1) thyroid nodule associated with a history of known primary malignant tumors, (2) cytology smears show benign thyroid follicular cells along with malignant cells. In one-half of the cases, no prior history of malignancy is available and the patients present with nodule in thyroid with an occult malignancy.[77] The cases of MC with predominant spindle cell component may simulate a metastatic fibrosarcoma. Hurthle cell tumor or follicular neoplasm may often mimic as metastatic renal cell carcinoma and vice versa. Metastatic giant cell carcinoma from lung or malignant melanoma may mimic anaplastic carcinoma of thyroid.

Differential Diagnosis
- Hurtle cell and follicular cell tumor
- Anaplastic carcinoma
- Spindle cell variant of MC.

Ancillary Techniques

Majority of the thyroid lesions can be diagnosed on the basis of the cytomorphological characteristics particularly PCT, MC and anaplastic carcinoma. In certain thyroid tumors such as follicular and Hurtle cell neoplasm, it is not possible to distinguish adenoma from carcinoma. Ancillary techniques in thyroid are unable to differentiate benign from malignant tumors. However, the ancillary techniques may help in confirmation of specific subtypes of thyroid carcinomas. The special techniques also help in the assessment of the prognosis of thyroid malignancies.

The common ancillary techniques used in thyroid tumors are:
1. Special stains
2. Immunocytochemistry
3. Flow cytometry
4. Molecular genetics

The common indications for ancillary technique to apply in the thyroid tumors are:
a. Diagnosis of primary thyroid malignancy
   i. Medullary carcinoma
   ii. Anaplastic carcinoma
   iii. Non-Hodgkin's lymphoma
b. Diagnosis of metastatic carcinoma
c. Diagnosis of parathyroid lesions

1. Special stains: Congo red stain is commonly used in thyroid FNAC to detect amyloid material.
2. Immunocytochemistry: The panel of immunocytochemistry helps in the identification of different primary and secondary malignancies of thyroid (**Table 26.5**). Immunocytochemistry should be preferably done on cell block preparation. Calcitonin positivity in case of MC and parathormone positivity along with TTF negativity in parathyroid neoplasm are diagnostic of these tumors. Anaplastic carcinomas are positive for vimentin and negative for thyroglobulin. To differentiate chronic LT and NHL, the demonstration of light chain restriction is helpful. However, this is necessary in selected cases only.
3. Flow cytometry: This is useful in the diagnosis of NHL. To differentiate a florid Hashimoto's thyroiditis and MALTOMA is a challenge to the cytopathologist. The demonstration of the light chain restriction is helpful in the diagnosis of

TABLE 26.5: Immunocytochemistry in thyroid aspirate

Tumors	*Antibody*	
	Positive	*Negative*
Medullary Carcinoma	Calcitonin is specific, Chromogranin, NSE synaptophysin and CEA	Thyroglobulin
Anaplastic carcinoma	Vimentin	Thyroglobulin
Papillary thyroid carcinoma	Thyroglobulin, TTF-1, CK7, CK19, EMA	CK20,
Insular carcinoma	Thyroglobulin, TTF-1	Calcitonin
Undifferentiated carcinoma	Vimentin, Pancytokeratin, Focal positivity for CEA and EMA	TTF-1, Thyroglobulin (usually negative)
Non-Hodgkin lymphoma	Light chain restriction (either kappa or lambda expression), CD19, CD20, CD3, CD5, CD23, CD10 (depending on the type of NHL)	
Parathyroid tumors	Parathyroid hormone	Calcitonin, TTF-1
Metastatic carcinoma	Cytokeratin, EMA	TTF-1, Thyroglobulin

Abbreviations: EMA, epithelial membrane antigen; TTF, thyroid transcription factor; CK, cytokeratin; NSE, neuron-specific enolase; NHL, non-Hodgkin's lymphoma; CEA, carcinoembryonic antigen

TABLE 26.6: Management of thyroid swelling after fine needle aspiration cytology (FNAC) report

Diagnostic category	*Management*
Group1: Benign lesions such as colloid goiter	Clinical follow-up should be done at 6 monthly to 1 year interval. USG in case of poorly palpable lesion
Group 2: Follicular lesions of undetermined significance or atypia of undetermined significance	• Serum TSH should be measured • If serum TSH is low, thyroid scan • If cold nodule, then surgical resection Or • FNAC should be repeated after 3–6 months. If repeat FNAC is also reported as atypical then surgical excision is advised
Group 3: Follicular neoplasm or Hurthle cell neoplasm	Surgical consultation for lobectomy or hemithyroidectomy
Group 4: Suspicious for malignancy	Surgical consultation for lobectomy
Group 5: Specific malignancy	Surgical consultation for lobectomy or hemithyroidectomy
Group 6: Inadequate	Re-aspirate under USG guidance, repeat FNAC after 3 months

Abbreviations: TSH, thyroid-stimulating hormone; FNAC, fine needle aspiration of cytology; USG, ultrasonography

B-NHL. Aberrant expression of T-cell markers suggests a case of T-NHL.

4. Molecular genetics: RET/PTC chromosomal rearrangements are commonly found in PTC. The demonstration of RET/PTC chromosomal rearrangement by RT-PCR for the diagnosis of PTC especially in the cases of difficult situation is fascinating.[78]

BRAF point mutations are the genetic hallmarks PTC and the demonstration of these mutations is also helpful in the confirmation of PTC.[79]

MANAGEMENT OF POST-FINE NEEDLE ASPIRATION CYTOLOGY

Diagnosis of Thyroid Lesion

The treatment and management of the thyroid nodule largely depend on the FNAC diagnosis of thyroid lesions and the broad category of the thyroid swelling.

Table 26.6 summarizes the post-FNAC management outline in brief as suggested by NCI sponsored group.[80]

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CHAPTER 27

Breast

ChapterContents

INTRODUCTION

Breast cancer is one of the most important public health problems in the world. It is one of the major causes of death due to cancer. It has been shown that the invasive carcinoma of breast passes through in situ carcinoma. There is considerable higher mortality and morbidity in the higher stage of invasive carcinoma of the breast. However, only 16% of breast carcinoma cases are diagnosed in stage I and majority of the women (54%) are diagnosed in stage II.[1] The palpable breast lesions are easily detected by the physicians. With the help of ultrasonography (USG) and mammogram, the nonpalpable breast lesions are also detectable and early stages of breast cancer can be recognized. However in a meta-analysis study, Gøtzsche PC and Olsen O did not notice any effect of mammographic screening on breast cancer mortality and they raised the validity of mammography as a screening test of breast cancer.[2] Other workers have highlighted the importance of mammographic test in breast cancer screening and till now it is considered as the best screening technique.[3] Newer other modalities particularly fine needle aspiration cytology (FNAC) is also very useful for the definitive diagnosis of breast cancer. Presently, FNAC is a popular technique and it is also a part of "triple test" for the diagnosis of breast lesions particularly breast carcinomas. FNAC has good diagnostic accuracy and has reduced the rate of the excision biopsy.[4]

INDICATIONS OF FINE NEEDLE ASPIRATION CYTOLOGY OF BREAST

Fine needle aspiration cytology is indicated almost all the palpable breast lesions and mammographically abnormal nonpalpable breast lesions. The major indications of FNAC of the breast lesion have been mentioned in **Box 27.1**.

There are several advantages of FNAC of breast lesion. These include (1) rapid diagnosis, (2) economically cost effective, (3) high diagnostic accuracy, (4) multiple sampling from the multiple areas of the breast, (5) avoids open surgery in inoperable malignancies, (6) performing ancillary techniques and (7) preoperative planning in malignant tumors. In addition, cytological diagnosis of benign lesion provides a rapid psychological relief to the patient.

CONTRAINDICATIONS

Truly speaking, there is no contraindication of FNAC of the breast. It is a safe procedure and the patient encounters minimal trauma. Needle tract seeding does not occur after FNAC technique. If adequate sterility is maintained then there is no chance of secondary infection. Hematoma after FNAC procedure may occur if adequate precaution is not taken after

BOX 27.1 Indications of fine needle aspiration cytology of breast

Diagnostic
- Benign versus malignant lesions of the breast
- Preoperative grading of breast carcinoma
- Hormone (estrogen and progesterone) and growth receptor (HER-2neu) evaluation
- Nonpalpable breast lesion with abnormal mammogram
- Inflammatory diseases of breast
- Recurrent tumor

Therapeutic
- Evacuation of cyst

BOX 27.2 Limitations of fine needle aspiration cytology (FNAC) of breast

- Inability to distinguish
  - DCIS from IDC
  - ADH from DCIS
  - Atypical cell in breast FNAC with indeterminate or gray zone area needs to have histopathology.
  - Inability to identify various low-grade carcinoma with certainty
- Certain benign breast lesions are difficult to subtype

Abbreviations: DCIS, ductal carcinoma in situ; IDC, infiltrating duct carcinoma; ADH, atypical ductal hyperplasia; FNAC, fine needle aspiration cytology

FNAC procedure. Pneumothorax may occur in case of FNAC of the axillary swelling. However, the conservative management is enough to manage such cases.

DIAGNOSTIC ACCURACY

Fine needle aspiration cytology of the breast is a good diagnostic technique because of high sensitivity and specificity. The diagnostic accuracy is to some extent operator dependent. However, the sensitivity of the technique ranges from 68% to 99%. The specificity of FNAC is as high as 99%.[5-7] The diagnostic accuracy of FNAC of breast increases if the cytopathologist performs FNAC and reports the same case. The false negative rate of FNAC is about 3–5%, however, it has been reported as high as 30%.[8]

These false negative cases can be easily avoided if the FNAC is done by an experienced cytologist and multiple sampling is done. Clinical history should also be considered and any clinically suspicious mass that are negative on cytology, should be repeated. False-positive rate in breast is near about 4%.[6,9]

In many centers, definitive surgery is done on the basis of FNAC report. Therefore, extreme care should be taken to avoid a false positive diagnosis. Medicolegal aspects of the false positive cases are also serious. Majority of the medicolegal cases are related with breast and mostly due to over diagnosis than under diagnosis.

LIMITATIONS OF FINE NEEDLE ASPIRATION CYTOLOGY

The cytologist should have adequate experience of FNAC technique. There may be many technical problems related to this procedure that can be easily avoided such as air drying of the smear, poor cellularity and improper sampling. However, there are certain inherent limitations of breast FNAC (**Box 27.2**). The major such limitation is inability to distinguish atypical ductal hyperplasia (ADH) from in situ carcinoma of the breast [ductal carcinoma in situ (DCIS)]. Similarly, it is very difficult to distinguish DCIS from infiltrating duct carcinoma (IDC) on the cytology smear.

BOX 27.3 False negative diagnosis of breast fine needle aspiration cytology (FNAC)

Sampling error
- Cystic lesion or necrosis
- Poor preservation and drying artifact
- Small focus of carcinoma in a benign lesion
- Scanty cellularity in deep seated lesion or fibrotic lesion
- Inadequate sampling from a large mass
- Interpretation error
- Certain types of carcinoma: tubular carcinoma, mucinous or colloid carcinoma, lobular carcinoma
- Well differentiated carcinoma
- Carcinoma arising from papillary tumor or fibrocystic disease

The false negative FNAC diagnosis of the breast may be due to sampling or interpretative error.[10] The major causes of sampling error may be due to necrosis, cystic change of the lesion or may be due to heterogeneous distribution of the tumor tissue such as small focal carcinomas in a large fibrocystic disease (FD) (**Box 27.3**). Low-grade carcinoma of breast and certain type of carcinomas such as lobular carcinoma, tubular carcinoma and papillary carcinomas are often difficult to diagnose on FNAC and may produce false negative diagnosis due to error in the interpretation.

The other major concerns are false positive diagnosis of breast FNAC that may give rise to medicolegal problems. The major reasons of false positive diagnosis are atypical cells in various reactive inflammatory lesions, fibroadenomas (FAds) with atypia, pregnancy and lactation induced changes in breast and ADH[11] (**Box 27.4**).

CLINICAL HISTORY

The detailed clinical history is helpful for diagnosis of cytology smear (**Table 27.1**). Malignancy is extremely uncommon in younger patients. Whereas, the presence of breast lump in any patient older than 45 years needs careful evaluation. Any

BOX 27.4 **False positive diagnosis of breast fine needle aspiration cytology (FNAC)**

- Fibroadenoma with reactive atypia
- Regenerative cells particularly histiocytes in inflammation
- Papilloma
- Fat necrosis
- Atypical ductal hyperplasia
- Sclerosing duct adenosis
- Pregnancy induced changes
- Gynecomastia with atypia
- Radiation induced changes
- Skin adnexal tumors

TABLE 27.1: Clinical comparison of malignant and benign breast lesions

Features	*Malignant*	*Benign*
Age	More than 45 years are suspicious	Younger patients
Duration	Recent origin	Long duration
Rapidity	Rapidly growing	Slowly growing
Swelling	Hard, fixed	Soft, mobile
Skin	Inflamed, ulcerated	Normal
Nipple	Recent retraction	Normal
Family history of breast Cancer	May be present	Absent
Mammogram	Microcalcification	Normal

hard, fixed lump with skin indurations is suspicious for cancer. Recent history of nipple retraction also indicates an underlying malignancy (**Fig. 27.1**) (**Table 27.1**). Any rapidly growing mass within a short period of time suggests a malignant neoplasm. However, mucinous carcinoma may have long history of the swelling. Family history of breast carcinoma is informative because certain breast carcinomas are hereditary. Treatment history of carcinoma of the breast is also helpful for proper interpretation of cytology smear.

TRIPLE TEST

The triple test was introduced by Johansen C in 1975 for the diagnostic evaluation of breast lump in elderly woman (more than 40 years). This test was introduced to avoid unnecessary open biopsy or frozen section.[12] This triple test includes mammogram, physical examination and FNAC. When all the three tests are positive then the lump should be considered as malignant. Similarly, when all the components of the triple test are negative then the lump is considered as benign. Triple test is highly accurate and it can avoid the necessity for open biopsy. However, frozen section or histopathological examination may be required if any of the components of the triad of triple test is negative.[13,14]

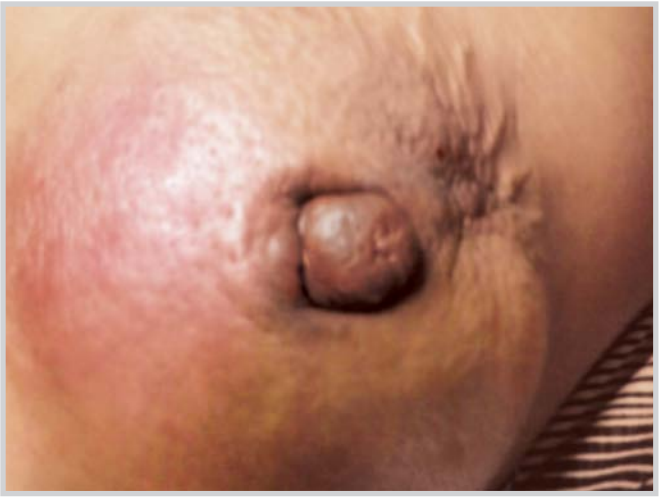

Fig. 27.1: Retracted nipple and puckered skin of the breast in a patient of carcinoma of breast

CORE NEEDLE BIOPSY VERSUS FINE NEEDLE ASPIRATION CYTOLOGY

Fine needle aspiration cytology is a simple, fast, cost effective and less invasive method for evaluation of breast lesions. However, despite its indisputable merit, FNAC has its own limitations such as high false-negative rates and equivocal results particularly to distinguish ADH from DCIS. To combat these limitations, core needle biopsy (CNB) has been introduced. CNB is becoming a popular technique among the pathologists for the diagnosis of breast lesions.[15] It is believed that CNB technique avoids FN cases and increases the diagnostic accuracy. However, Park SM et al. showed that the false negative rate is marginally different in FNAC (6.2%) versus CNB (9%). The sensitivity and specificity of FNAC in breast lesions are 93.8% and 80.8%, respectively, whereas, the sensitivity and specificity of CNB are 92.1%, 90.9%.[16] Other studies also showed marginal increase in sensitivity and specificity in CNB versus FNAC.[17,18]

Core needle biopsy has also certain disadvantages. It is a time consuming procedure and requires anesthesia. The result of CNB may show considerable variation depending on the needle size, number of cores and operator experience. **Table 27.2** has compared CNB and FNAC technique.

In fact, the choice between CNB versus FNAC depends to some extent on the experience of the cytopathologist. Radiological guided FNAC may reduce the inadequacy and false-negative rate. A combined use of CNB and FNAC gives much better result and many people suggest that CNB and FNAC are complementary to each other.[19,20]

ADEQUACY OF THE SAMPLE

Adequacy criteria of FNAC of breast sample are to some extent depends on the laboratory and the operators who perform FNAC. The National Cancer Institute (NCI)—sponsored conference in Bethesda on uniform approach of breast did not recommend any specific number of cells for adequacy of breast FNAC.[21] They considered a sample adequate that leads to resolution of

TABLE 27.2: Comparison of core needle biopsy (CNB) and fine needle aspiration cytology (FNAC)

Features	*FNAC*	*CNB*
Time to take	Rapid	Time consuming
Anesthesia	Not required	Required
Multiple sampling	Possible	Not possible
Cost	Cheap	Costly
Sensitivity and specificity	Low	Slightly high
Gray zone lesion (ADH, DCIS)	Not easily detectable	Detectable

Abbreviations: DCIS, ductal carcinoma in situ; ADH, atypical ductal hyperplasia; FNAC, fine needle aspiration cytology; CNB, core needle biopsy

BOX 27.5 **Adequacy of breast fine needle aspiration cytology (FNAC)**

- No specific minimal cell number
- Aspirator should judge whether the FNAC report is consistent with the clinical or radiological findings
- Cytologists should judge the smear quality such as air drying, artifact, etc
- Cytologists should mention the amount of epithelial cells
- Individual laboratories may decide their own guidelines about the number of cells for adequacy

Layefield's proposal of adequacy criterion

Six or more cell clusters (cumulative total) or the presence more than 10 intact bipolar cells per 10 medium-power fields (× 200)

a problem presented by a lesion in a particular patient's breast and no minimal quantity of cells is required for adequacy. The cytologists should determine the adequacy of the sample based on the cytologic and clinical findings of individual cases. The pathologists should also judge the smear regarding any significant distortion and artifact. According to NCI conference, the cytopathologist should always mention about the quantity of the epithelial cells on the smear such as:

- *Scanty cellularity*: Only occasional epithelial cell clusters present
- *Moderate cellularity*: Readily identifiable clusters on the smear
- *Abundant cellularity*: Multiple clusters of epithelial cells in every field.

Layfield et al. believes that the stringent criterion on adequacy is required to reduce the number of false negative diagnosis because majority of the false-negative sample is due to inadequate cellularity.[22] They suggested a cut-off point for satisfactory smears at a level of six or more cell clusters (cumulative total) or the presence more than 10 intact bipolar cells per 10 medium-power fields (× 200) (**Box 27.5**).

In case of cystic lesions of breast, there is no strict criterion about the number of epithelial cells on the smear. However, after evacuation of the cyst any residual solid mass should be reaspirated.

HISTOLOGY OF BREAST

Breasts are modified apocrine sweat glands. Breasts develop embryologically along the milk line that extends from the thorax to groin. Therefore, accessory breast may be found anywhere along the milk line. Each breast consists of 15–20 lobes radiating out from the nipple. These lobes are separated by collagenous septae. Each lobe has its own duct and before opening on the surface the duct is dilated to form the lactiferous sinus. The main duct divides within each lobe of the breast and ultimately forms multiple terminal ducts. Each terminal duct is connected with lobule and each lobule consists of multiple acini. The combined terminal duct and lobule is known as *terminal duct lobular* unit. The duct and acini of the breast are lined by luminal layer of columnar to cuboidal epithelium and basal layer of myoepithelial cells.

NORMAL CYTOLOGY OF BREAST

Fine needle aspiration cytology (FNAC) of the normal breast usually yields scanty cellularity. The following cells are seen in FNAC of a non-neoplastic breast.

- *Ductal cells* (**Fig. 27.2**): These are found singly or honeycomb like flat cluster. Ductal cells are small round cells with monomorphic nuclei. The individual cells have regular nuclear margin with homogenous chromatin and inconspicuous nucleoli.
- *Myoepithelial cells* (**Fig. 27.3**): These are easily identifiable bipolar naked cells. Occasional cells may have scanty preserved cytoplasm.
- *Apocrine cells* (**Fig. 27.4**): These are large cells with abundant granular eosinophilic cytoplasm. The nucleus of the cell is central to eccentric in position, round and monomorphic.
- *Foam cells* (**Fig. 27.5**): The foam cells have abundant vacuolated cytoplasm and centrally placed monomorphic round nucleus. Phagocytosed material may be found in the cytoplasm. The presence of foam cells usually indicates cystic degeneration.
- In addition, FNAC of the breast may also yield fat and stromal fragment.

BENIGN LESIONS OF THE BREAST

The **Box 27.6** shows the classification of the benign lesions of breast.

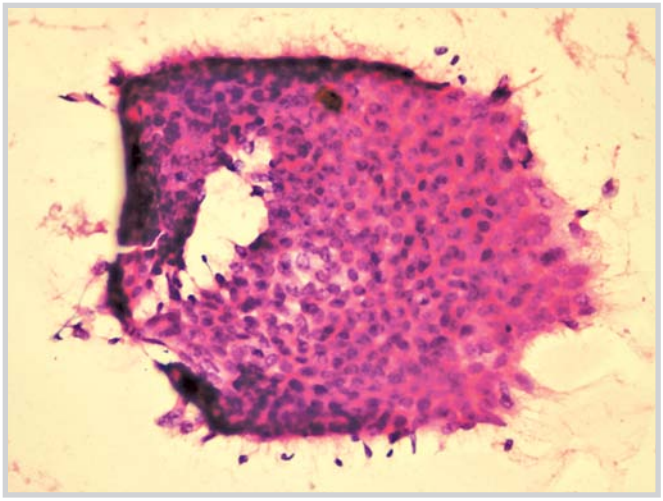

Fig. 27.2: Benign ductal cells in tight cluster [hematoxylin and eosin (H & E) × medium power (MP)]

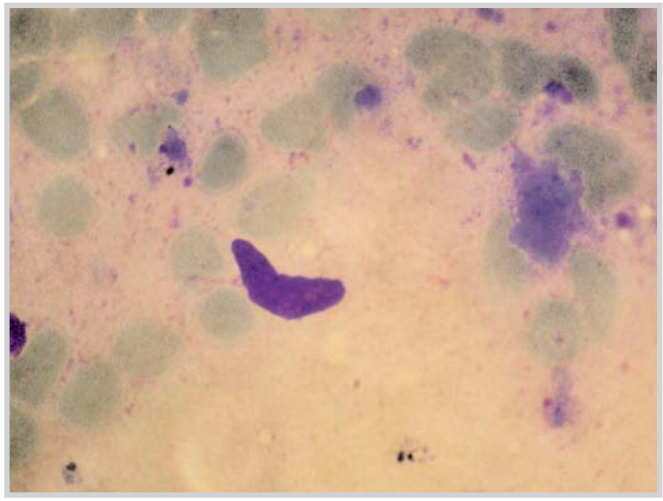

Fig. 27.3: Naked bipolar myoepithelial cell [May-Grünwald-Giemsa (MGG) × high power (HP)]

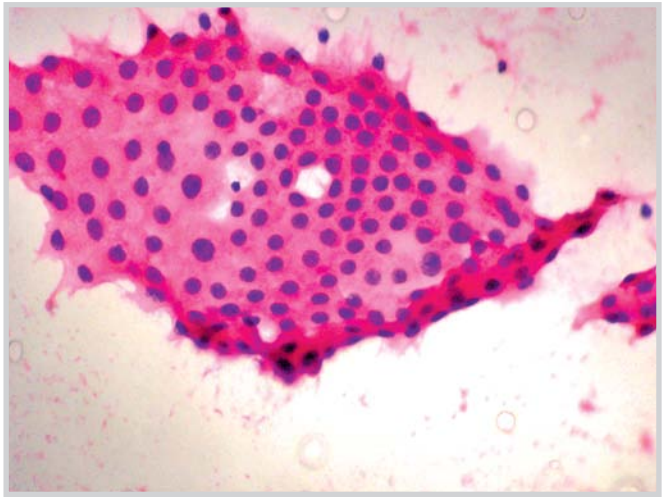

Fig. 27.4: Large cluster of apocrine cells with moderate to abundant cytoplasm and centrally placed monomorphic nuclei (H & E × HP)

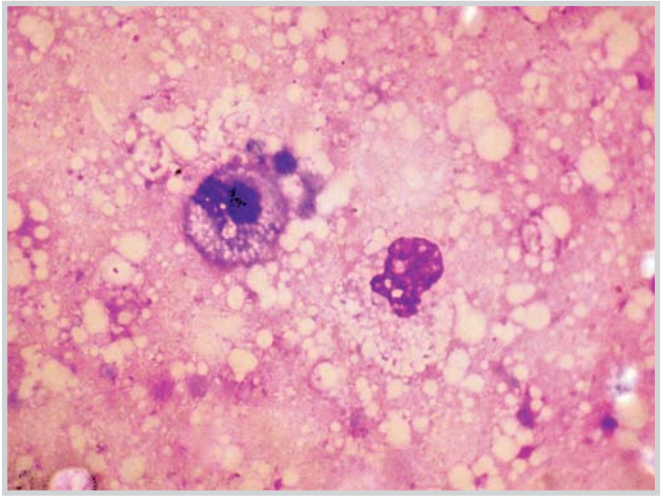

Fig. 27.5: Foamy histiocytes indicating degenerative changes (MGG × HP)

BOX 27.6 Benign breast lesions

Inflammatory lesions
- Acute inflammation
- Chronic inflammation
- Granulomatous mastitis

Lesions caused by trauma
- Fat necrosis
- Lesions caused by breast augmentation or reduction surgery

Benign proliferative breast disease
- Fibroadenoma
- Fibrocystic disease
- Lactating adenoma
- Granular cell tumor
- Intraductal papilloma
- Atypical ductal hyperplasia

Inflammatory Lesions

Mastitis

It is the inflammation of breast. Mastitis usually occurs in postpartum lactating woman. The bacteria invade the breast through small cracks of the nipple. The common organisms are *staphylococci* and *streptococci*. The patient presents with swelling, pain and tender mass in the breast. Mastitis may mimic clinically as inflammatory carcinoma.

Cytology

Fine needle aspiration cytology of the cases of mastitis usually yields pus. The cytological picture depends on the chronicity of the inflammation. In acute mastitis, the smears show abundant polymorphs, necrotic debris and occasional ductular cells. In subacute and chronic mastitis, the smears show increased proportion of lymphocytes, plasma cells, histiocytes and clusters

of ductular cells. The ductal cells may show reactive nuclear atypia due to chronic inflammation.

Differential Diagnosis
Inflammatory carcinoma: Reactive nuclear atypia of ductal cells may mimic carcinoma.

Granulomatous Mastitis

Granulomatous mastitis (GM) is an uncommon breast lesion. The clinical and radiological features of GM often mimic malignancy. The patients usually present as hard breast lump. The patients are in reproductive age period and are often lactating. The etiology of GM is mainly idiopathic of unknown etiology. The specific causes of GM are tuberculosis, sarcoidosis, fungal infections or foreign body granulomas such as due to leakage of silicone implant or suture granuloma.[23]

Cytology (Fig. 27.6)
Fine needle aspiration cytology (FNAC) of GM shows multiple epithelioid cell granulomas, reactive lymphoid cells, multinucleated giant cells, necrosis and occasional clusters of ductular cells (**Box 27.7**). In addition, there may be specific causes of GM such as suture material, fungal profiles or acid-fast bacteria (AFB) in Ziehl-Neelsen (Z-N) stain.

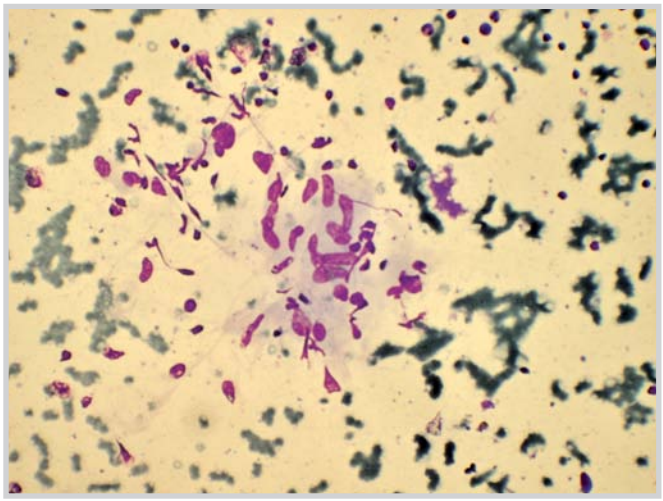

Fig. 27.6: Epithelioid cell granuloma in a case of granulomatous mastitis (MGG × MP)

BOX 27.7 Granulomatous mastitis

- Multiple epithelioid cell granulomas
- Lymphocytes and capillary fragments
- Multinucleated giant cells
- Necrosis
- Reactive ductal epithelial cells

Differential Diagnosis
Low-grade IDC is the main differential diagnosis of GM.

Chronic Subareolar Abscess

This is a specific clinical condition characterized by chronic infection of the lactiferous sinus or duct and the formation of subareolar abscess. The chronic inflammatory reaction often causes squamous metaplasia of the duct lining epithelium and blockage of the duct lumen due to keratin plugging. This abscess often bursts out on the surface and forms fistula in the nipple.

Cytology
Fine needle aspiration cytology of the subareolar abscess shows abundant polymorphs, lymphocytes, histiocytes, multinucleated giant cells and metaplastic squamous cells. In addition, the smears also show reactive atypical ductal cells.[24,25]

Fat Necrosis

Fat necrosis clinically mimics malignancy. It may occur due to direct trauma to the breast or as an effect of local radiation in a carcinoma of breast. The presence of fat necrosis does not exclude the possibility of an underlying malignancy. The patient typically presents as hard lump in the breast fixed to the adjacent tissue.

The salient diagnostic points of fat necrosis are described in **Box 27.8**.

Cytology (Fig. 27.7, Figs 27.8A and B)
Fine needle aspiration cytology of the fat necrosis yields amorphous material. The smears show dirty degenerated fat (**Box 27.8**). The nuclei of the fat cells are often enlarged. In addition, the smear shows mixed inflammatory cells and large atypical histiocytes. The enlarged and mildly pleomorphic histiocytes may mimic malignant cells. Occasional ductal cells show mild nuclear enlargement.

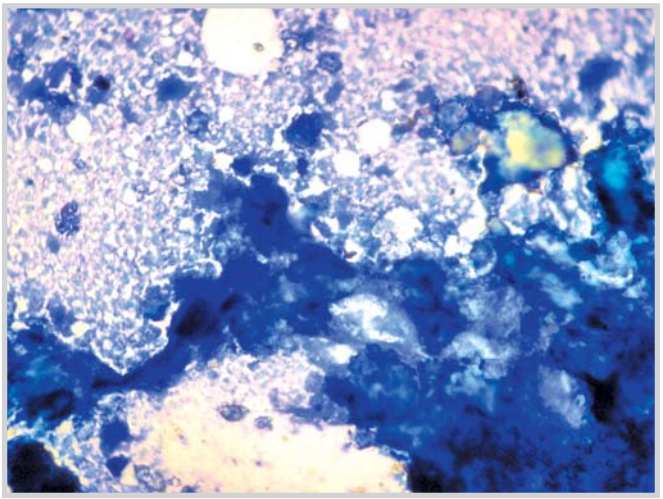

Fig. 27.7: Degenerated necrotic fat and calcification in fat necrosis of breast (MGG × MP)

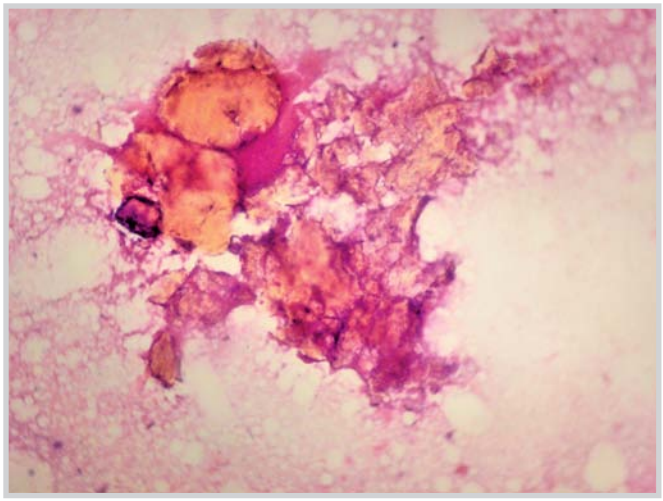

Fig. 27.8A: Necrotic fat cells and calcified material in fat necrosis (H & E × HP)

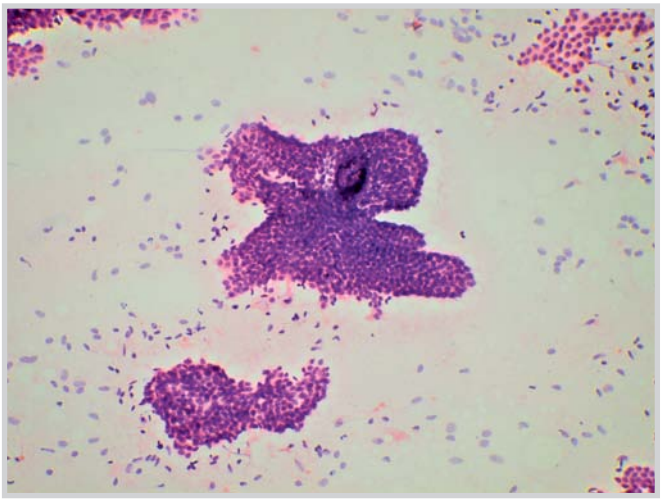

Fig. 27.9A: Stag horn like clusters of ductular cells in fibroadenomas of breast (H & E × MP)

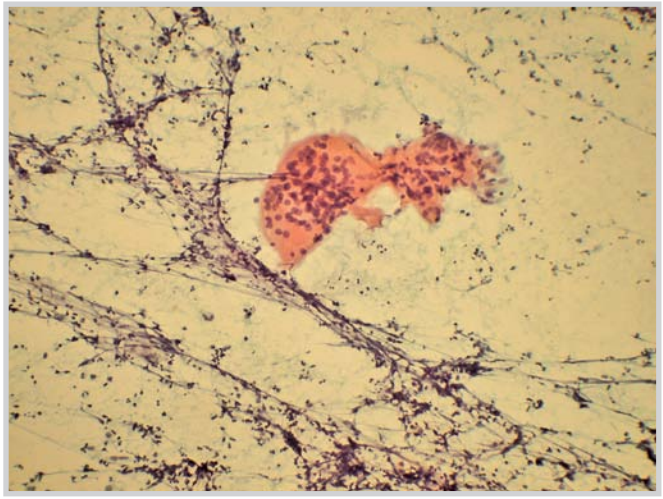

Fig. 27.8B: Inflammatory cells and multinucleated giant cells in fat necrosis (Papanicolaou's stain × MP)

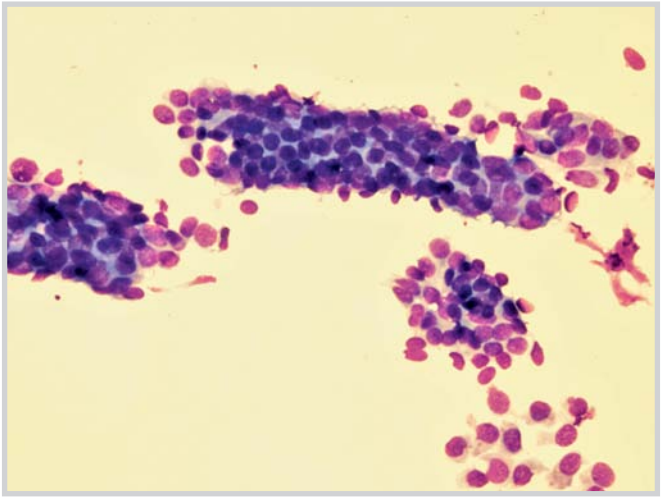

Fig. 27.9B: Fibroadenomas of breast: ductular cells in tight clusters along with bipolar cells (MGG × MP)

BOX 27.8 **Fat necrosis**

- Neutrophils and lymphocytes
- Multinucleated giant cells
- Necrotic fatty background.
- Epithelioid cell granuloma

D/D: Carcinoma.

Abbreviation: D/D, differential diagnosis

Benign Noninflammatory Lesions

Fibroadenoma

Fibroadenoma is the most common benign breast tumor of the breast. FAd may occur in any age group. However, it is typically noted between 20 and 35 years of age. The presence of FAd in prepubertal and postmenopausal women is rare. The patient usually presents with a well-circumscribed, firm to soft, mobile mass in the breast.

Cytology (Figs 27.9A and B to 27.12A and B)

FNAC of the FAd yields thick sticky material. The cytology smears show abundant cellularity (**Box 27.9**). Striking biphasic pattern is seen in most of the cases. The smears show both round to oval ductal cells and spindle bipolar cells. The ductal cells are present in multiple tight clusters, and in three-dimensional (3D) antler horns like arrangement. Single dissociated duct cells are also seen. Individual cells have round monomorphic nuclei with fine granular chromatin and small nucleoli. Chondromyxoid stromal material is also noted. This gives bright magenta colored appearance in May-Grünwald-Giemsa (MGG) stain. Clusters of stromal cells are embedded in the pinkish connective tissue stroma. The stromal cell shows scanty cytoplasm and spindle

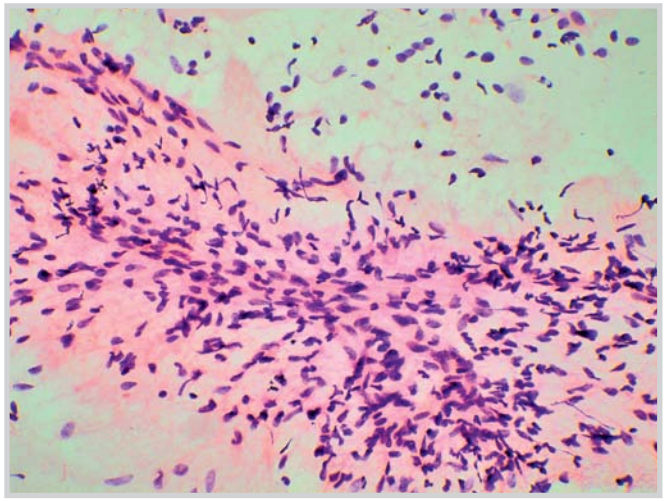

Fig. 27.10: Oval to spindle-shaped stromal cells embedded in connective tissue stroma in fibroadenomas of breast (H & E × HP)

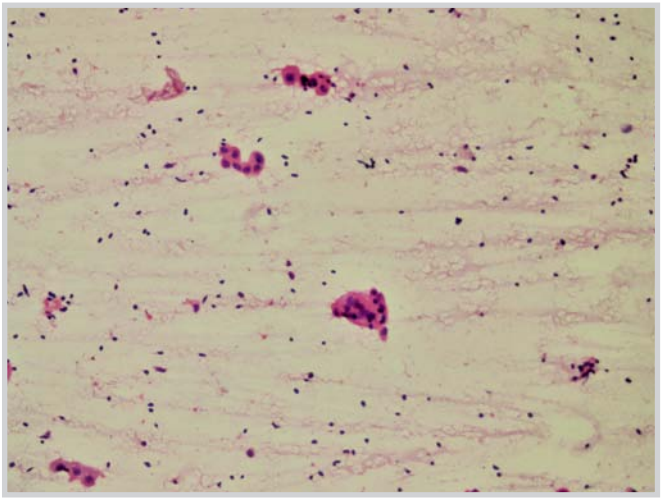

Fig. 27.12A: Fibroadenoma: apocrine cells and multinucleated giant cells [H & E × low power (LP)]

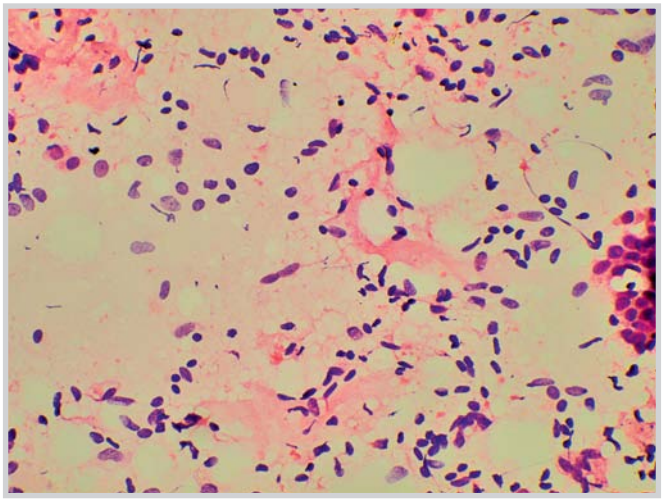

Fig. 27.11A: Discrete ductular cells and many bipolar stromal cells in fibroadenomas of breast (H & E × HP)

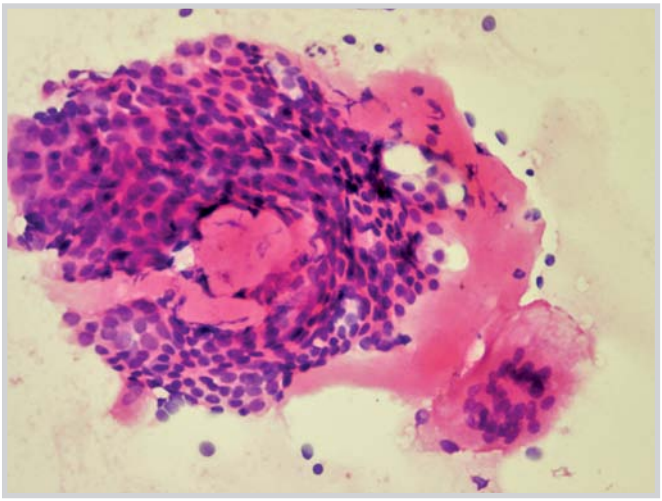

Fig. 27.12B: Fibroadenoma: multinucleated giant cells in higher magnification (H & E × HP)

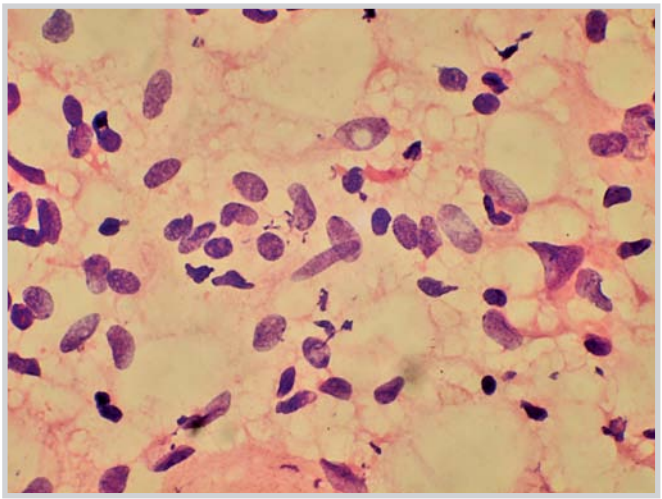

Fig. 27.11B: Higher magnification showing better morphology of ductular cells in fibroadenomas of breast [H & E × oil immersion (OI)]

BOX 27.9 Fibroadenoma

- Abundant cellularity
- Sheets of cells
- Antler horns like tight clusters
- Biphasic pattern
- Ductal cells
  - Round, monomorphic
  - Fine granular chromatin
  - Small nucleoli
- Bright magenta colored stromal material
- Many bipolar spindle shaped cells
- Naked nuclei
- Occasional foam cells and apocrine cells

shaped nuclei. Naked bipolar myoepithelial cells are easily recognizable in Papanicolaou's staining.

There may be various changes in FAd such as:

- Nuclear atypia
- Apocrine metaplasia
- Foam cells
- Mucinous metaplasia
- Bony metaplasia
- Cartilaginous metaplasia.

Out of these, the most significant change is nuclear atypia of ductal cells. This atypia in FAd may be due to hormonal stimulation or inflammation. Atypia is one of the causes of false positive diagnosis in breast FNAC. In most of the times, histopathology of such cases reveal benign lesion. Well-maintained polarity of the cells, abundant naked bipolar cells in the background and essentially normal chromatin pattern of the atypical cells are suggestive of FAd. Mild atypia in a smear of FAd should be overlooked.[26,27]

The presence of tight papillary clusters of ductal cells in FAd may simulate papillary tumors. However, FAd does not show typical fibrovascular core as noted in papillary tumors. In addition, the multiple stromal fragments are more often seen in FAd. It is very difficult to distinguish FAd and cytosarcoma phylloides (CP) in FNAC smear. In CP, there is relative abundance of stromal component over the epithelial component. The stroma is also more cellular in CP than in FAd. FNAC smear of CP also shows numerous long plump spindle-shaped cells along with large folded sheets of epithelium.[28,29] However, at times FAd may show large stromal fragments and may be difficult to distinguish from CP on the basis of cytological features.[30]

Differential Diagnosis

- Cytosarcoma phylloides tumor
- Low-grade IDC
- Atypical ductal hyperplasia
- Papillary neoplasm.

Fibrocystic Diseases

Fibrocystic disease (FD) is extremely common lesion in adult female and about 60–90% women are affected by FD in their life time. This disease is most frequently seen in the patients of 25–45 years age. The FD is usually bilateral and multifocal, however, one breast may be affected more than the other one. The patient presents with breast lump that shows variation in size and symptoms according to hormonal fluctuation of the menstrual cycle. The clinical and radiological features of FD may simulate carcinoma and the presence of FD makes the detection of carcinoma by mammography more difficult.

Cytology (Figs 27.13A and B)

FNAC in predominant fibrous type of FD yields scanty material. However, in cystic type of FD, FNAC yields clear to turbid fluid. Discrete and large monolayer clusters of apocrine cells are often seen (**Box 27.10**). These cells have abundant granular eosinophilic cytoplasm. The nuclei of the apocrine cells are in central position. Nuclei may show mild enlargement and pleomorphism. The smears also show many foamy histiocytes, and small clusters of benign ductal cells. The ductal cells are usually in monolayer sheets. The cells have monomorphic nuclei with fine granular chromatin and inconspicuous nucleoli. The smear also shows scattered naked bipolar cells, stromal fragments and adipose tissue. Occasionally, it is difficult to distinguish FAd from FD due to the relative abundance of ductal cells in the FD. However, large fragments of stromal cells, abundant bipolar spindle cells and antler horn like cells are characteristically seen in FAd

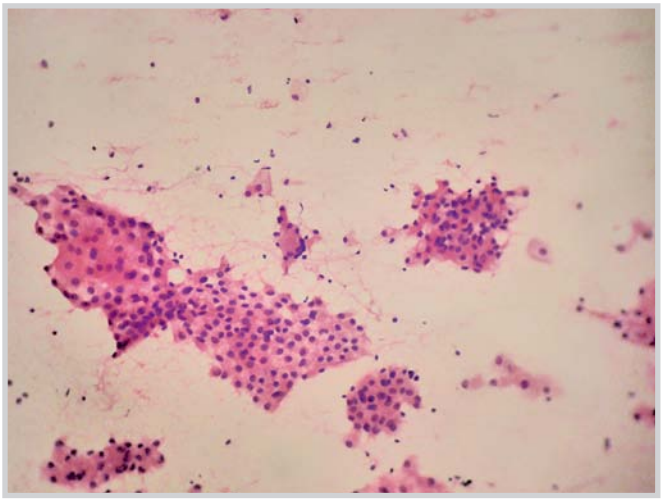

Fig. 27.13A: Fibrocystic disease of breast: clusters of apocrine cells and foamy histiocytes (H & E × MP)

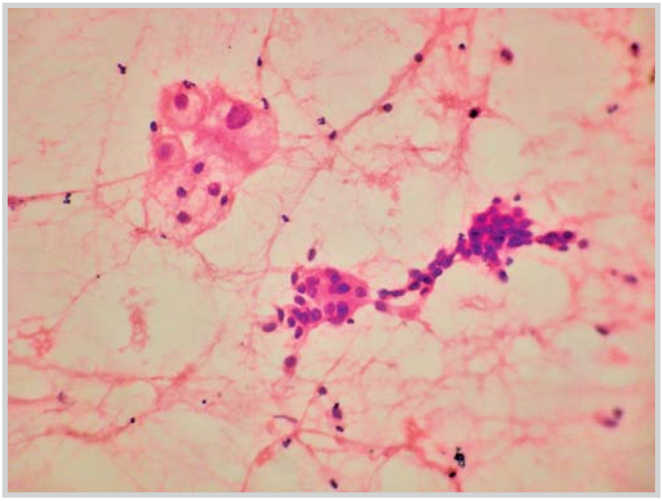

Fig. 27.13B: Fibrocystic disease of breast: foamy histiocytes and benign ductal cells (H & E × HP)

BOX 27.10 Fibrocystic diseases

- Apocrine cells
- Foam cells
- Benign monomorphic ductal cells
- Mild anisocytosis of ductal cells
- Varying amount of stromal fragments and myoepithelial cells present
- Varying amount of inflammatory cells
- Debris of cyst fluid

TABLE 27.3: Distinguishing points between fibroadenoma and fibrocystic change

	Fibroadenoma	*Fibrocystic disease*
Swelling	Well-circumscribed, mobile	Ill-defined, diffuse, firm area
Location	Usually unilateral	Bilateral, multifocal
Hormonal dependency	No	Variation of size and symptoms depending on menstrual cycle
Aspirate	Particulate	Usually, clear or turbid fluid
Ductal cells	Abundant	Relatively scanty
Ductal cell arrangement	Antler horn-like 3D clusters	Monolayer sheets
Naked bipolar myoepithelial cells	Abundant	Scanty
Chondromyxoid stromal fragments	Abundant	Scanty to absent
Apocrine cells	Scanty	Relatively more

(**Table 27.3**). Mild degree of nuclear enlargement may be noted in ductal cells due to fluid effect. At times, the atypical ductal cells may create diagnostic difficulty with a low-grade carcinoma. Chromatin pattern of the cell is helpful in this condition.

Differential Diagnosis

- Fibroadenoma
- Atypical ductal hyperplasia
- Carcinoma

Galactocele

Glactoceles are small cysts filled with milk due to occlusion of the lactiferous duct. Aspirate yields milky fluid. FNAC smear shows (**Fig. 27.14**) large cells with abundant vacuolated cytoplasm in a milky background.[31,32]

Lactating Adenoma and Lactational Breast

Lactating adenoma is well-circumscribed freely mobile solitary or multiple mass and characteristically seen in pregnant or lactating patients. This is actually the localized area of hyperplasia in the lactating breast.

Cytology (Figs 27.15 and 27.16)

To distinguish lactating adenoma and lactational changes in breast is difficult in FNAC smear as both the lesions show similar cytomorphological features. FNAC yield milky white fluid. Smears show discrete or loosely cohesive ductal epithelial cells in a proteinaceous dirty background (**Box 27.11**). Individual cells are large and filled with multiple vacuoles. The nuclei are round, monomorphic and hyperchromatic with single prominent nucleoli. Due to the breakdown of the fragile cytoplasm of the cells, the smear may

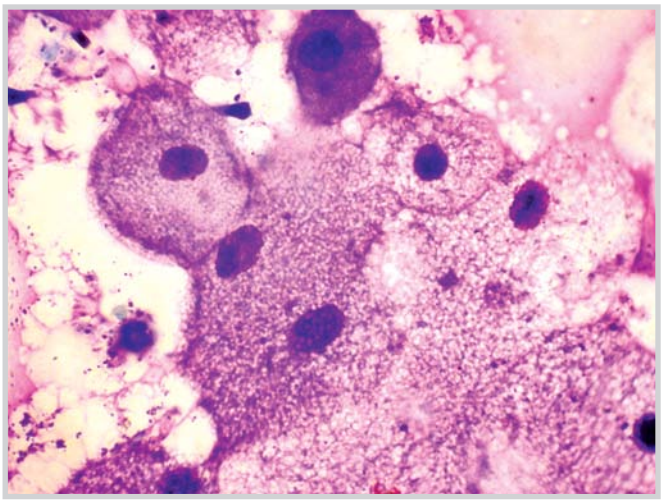

Fig. 27.14: Large cells with abundant vacuolated cytoplasm in a milky background in a case of galactocele of breast (MGG × HP)

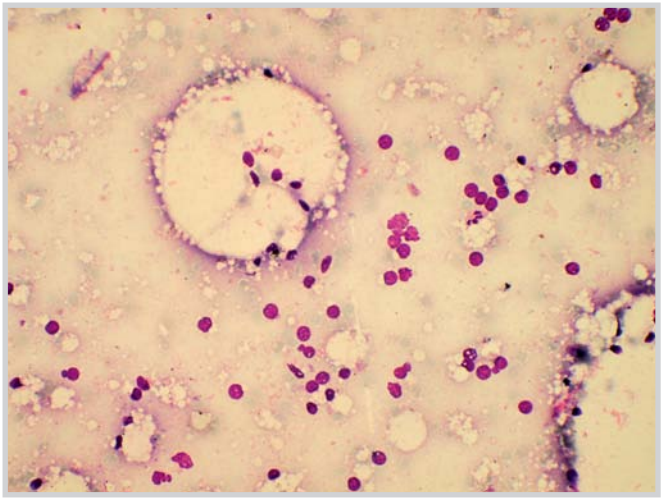

Fig. 27.15: Many discrete cells in a milky fatty background (MGG × MP)

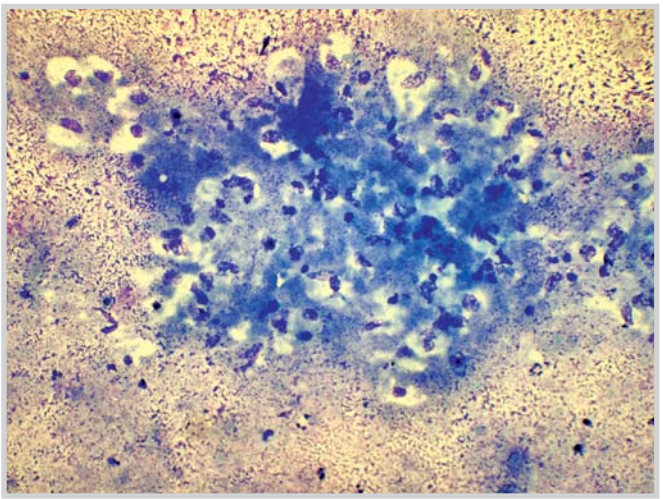

Fig. 27.16: Abundant foamy galactophages in a milky fatty background in a lactating adenoma of breast (MGG × HP)

BOX 27.11 Lactational adenoma or lactating breast

- Milky white fluid
- Discrete or loosely cohesive ductal epithelial cells
- Large cells filled with multiple vacuoles
- Enlarge round hyperchromatic nuclei with single prominent nucleoli
- Numerous naked nuclei
- Many polymorphs, lymphocytes and macrophages
- Proteinaceous dirty background

show many naked nuclei. In addition, the smears may also show many polymorphs, lymphocytes and macrophages. The proteinaceous and frothy dirty background of the smear may obscure the detailed cytomorphology of the individual cells.

Discrete cells with nuclear pleomorphism and prominent nucleoli may mislead the cytologists to a wrong diagnosis of carcinoma. This wrong diagnosis can be avoided if the history of lactation is known. The nuclear abnormality of carcinoma is much greater than that of lactating breast.

Differential Diagnosis

- Carcinoma in pregnancy or lactation: It is often very difficult to distinguish carcinoma in pregnancy or lactation with pregnancy associated changes. However, nuclear characters such as nuclear pleomorphism and angulated irregular thickened nuclear margin indicate malignancy.
- Fibroadenoma
- Galactocele.

Proliferative Breast Disease

Benign epithelial lesions of the breast are classified on the basis of relative risk to develop carcinoma. The lesions, those are not associated with the risk of development of cancer, are known as *non-proliferative breast disease* (non-PBD) and rest of the lesions are known as *proliferative breast disease* (PBD). The PBD without atypia is associated with 1.5–2 fold relative risk of developing carcinoma whereas PBD with atypia is associated with almost 4–5 folds higher risk of developing carcinoma.[33] PBD without atypia includes sclerosing adenosis, collagenous spherulosis, papillomatosis and ductal hyperplasia.

World Health Organization (WHO) working group favors to maintain the traditional classification of epithelial hyperplasia as such (**Box 27.12**):

BOX 27.12 Traditional classification of epithelial hyperplasia

- Usual ductal hyperplasia
- Flat epithelial atypia
- Atypical ductal hyperplasia
- Ductal carcinoma in situ grade 1
- Ductal carcinoma in situ grade 2
- Ductal carcinoma in situ grade 3

It is often difficult to distinguish these lesions on FNAC material. PBD shows large number of cohesive ductal cells along with naked nuclei. Here, the ductal cells are predominantly arranged in monolayer sheets. Occasional clusters of cells with mild nuclear crowding and pleomorphism are also seen. Cells show fine granular nuclear chromatin and inconspicuous nucleoli.

Proliferative Breast Disease with Atypia

As already mentioned, PBD is divided into three grades mild, moderate and severe hyperplasia.[34]

- Grade 1: No or mild hyperplasia: There is no risk for carcinoma.
- Grade 2: Moderate or florid hyperplasia: 1.5–2 times risk for carcinoma.
- Grade 3: Atypical ductal or lobular hyperplasia: 4–5 times risk for carcinoma.

In addition, another group was included in this category:
Grade 4: Ductal or lobular carcinoma in situ.[35]

Diagnosis of PBD with atypia or ADH is primarily based on following criteria:

- Cellularity
- Cell arrangement
- Cellular pleomorphism
- Myoepithelial cells
- Nuclear pleomorphism
- Nucleoli
- Chromatin clumping.

Salient cytological features are[33,36-40] (**Box 27.13**):

Cytology (Figs 27.17 to 27.19)

FNAC smear of ADH is rich in cellularity. The cells are arranged in large, complex multilayered sheets. The myoepithelial cells are closely intermingled with the cluster of ductal cells. Pseudopapillary arrangement of ductal cells is often seen. Occasionally, the cells are arranged as cribriform like pattern with slit like spaces in between. There is nuclear crowding and loss of polarity of the cells. The cells show distinct cytoplasmic borders and pleomorphism. The nuclei are enlarged, pleomorphic with coarse chromatin and prominent nucleoli. Loosely, cohesive small clusters of cells and occasional dispersed ductal cells are also seen. These cytological features of ADH may be mistaken as IDC. However, the presence of myoepithelial cells, clustering and cohesion of cells and lack of significant number of isolated single cells are the important features to distinguish ADH from IDC.

The diagnosis of ADH is a challenge to the cytopathologist as the cytomorphology of this lesion is intermediate between IDC

BOX 27.13 Hall marks of proliferative breast disease with atypia or atypical ductal hyperplasia (ADH)

- Large complex multilayered clusters
- Crowding and loss of polarity of nuclei
- Cellular pleomorphism
- Nuclear anisocytosis
- Irregular coarse chromatin and prominent nucleoli
- Presence of myoepithelial cells

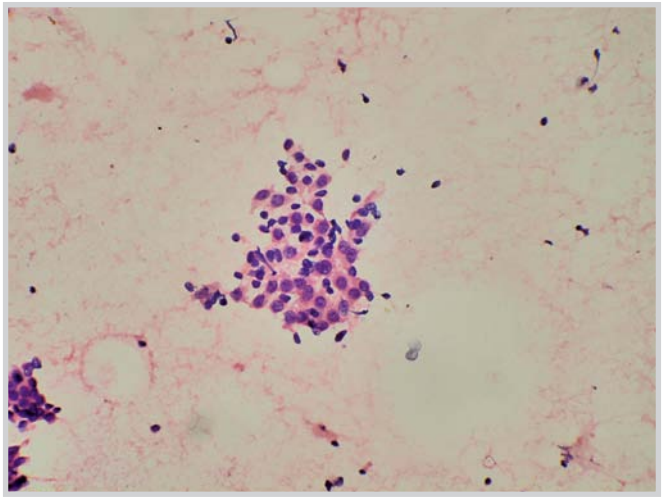

Fig. 27.17: Mono-layered cluster of ductular cells with mild nuclear atypia in proliferative breast disease with mild atypia (H & E × MP)

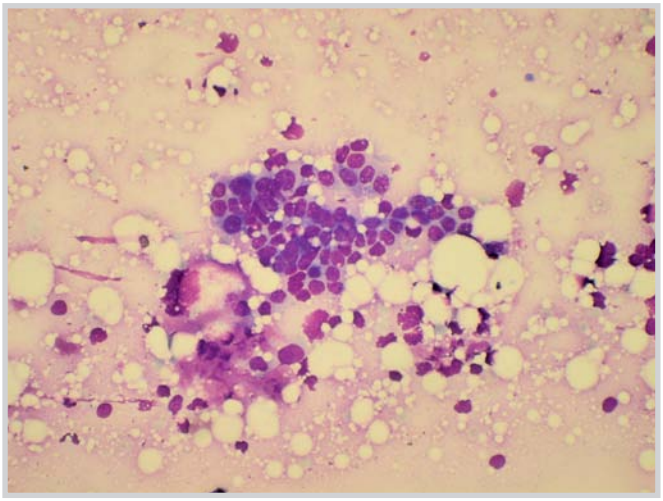

Fig. 27.18: Cluster and discrete ductal cells with nuclear overlapping and moderate nuclear pleomorphism (MGG × MP)

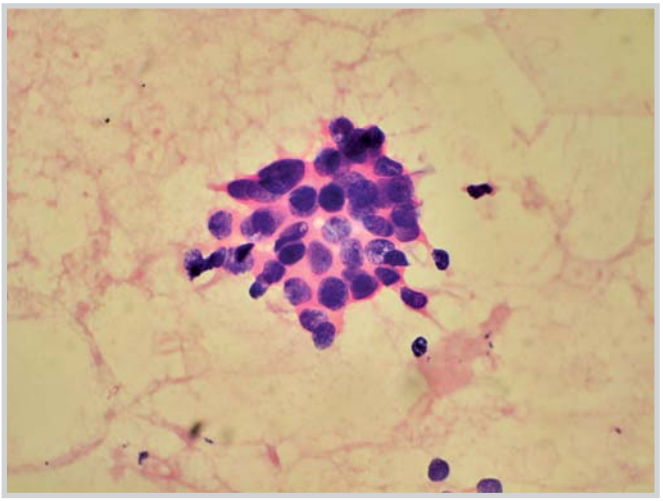

Fig. 27.19: Proliferative breast disease (PBD) with atypia showing nuclear overlapping and pleomorphism (H & E × MP)

and benign lesion. The distinction of these lesions has important clinical implications. To combat the challenge Massod et al. proposed a scoring system based on cellular arrangement, cellular pleomorphism, anisonucleosis, nucleoli, nuclear chromatin, and the presence of myoepithelial cells. Each feature is scored from 1 to 4 and the final diagnosis depends on total scoring.[41]

Differential Diagnosis

- Fibroadenoma: Cellular FAd may be difficult to distinguish from PBD. The key cytological features of FAd are:
  - Antler horn clusters
  - Flat or monolayered sheets
  - Fragments of fibromyxoid stroma.
- Ductal carcinoma in situ: Discussed below.
- Proliferative breast disease without atypia: It is important to distinguish PBD without atypia from ADH. **Table 27.4** describes the salient points.

Ductal Carcinoma In Situ[36,42]

Ductal carcinoma in situ is a heterogeneous group of lesion with variable architectural, histological and natural history. DCIS is characterized by marked epithelial proliferation with mild to marked nuclear atypia. The chance of progression of DCIS to invasive cancer depends on several factors such as histological subtype, grade and also extent of the tumor. Due to increasing use of mammography the incidence of DCIS has increased significantly. DCIS represents almost 25% of breast cancer in screened population.[42] The vast majority of DCIS is detected by screening mammography and only a few cases present with clinical symptoms such as palpable swelling, nipple discharge and nipple retraction.

Cytology (Figs 27.20 and 27.21)

It is extremely difficult to recognize this entity on cytology smear. The cytological features depend on noncomedo versus comedo variety of DCIS (**Box 27.14**). FNAC smears of comedocarcinoma show predominantly cohesive clusters and also discrete malignant cells with background necrotic cell debris and calcified

TABLE 27.4: Cytologic features of proliferative breast disease without atypia and atypical ductal hyperplasia (ADH) of breast

Cytology	*Proliferative breast disease without atypia*	*ADH*
Cellularity	Moderate	Abundant
Cell arrangement	Crowded sheets with regular nuclear spacing	Three-dimensional crowded sheets with nuclear overlapping
Nuclear pleomorphism	Absent	Present, mild to moderate
Nucleoli	Inconspicuous	Prominent
Hyperchromasia	Absent	Present
Chromatin	Fine	Coarse

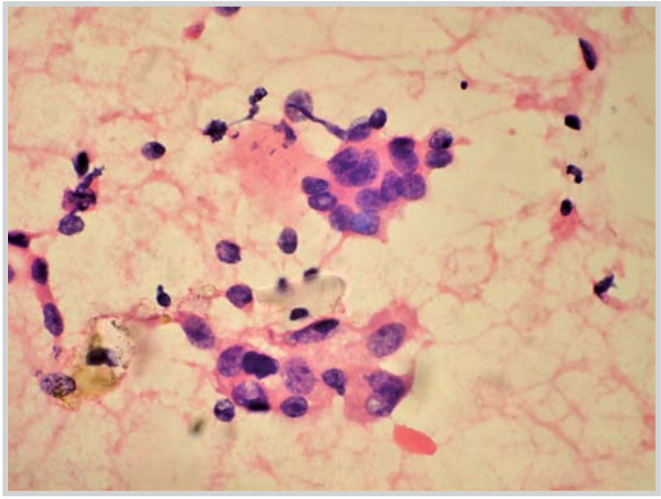

Fig. 27.20: Loose clusters of cells with marked nuclear pleomorphism in carcinoma in situ. Note the clean background (H & E × MP)

BOX 27.14 Ductal carcinoma in situ

Comedo type
- Cohesive clusters and also discrete malignant cells
- Necrotic cell debris
- Calcified material
- Moderate nuclear enlargement
- Nuclear pleomorphism
- Mitotic activity

Noncomedo type
- Solid, papillary and cribriform clusters
- Relatively monomorphic, small to intermediate in sized cells
- Round nuclei and small nucleoli
- No myoepithelial cells.

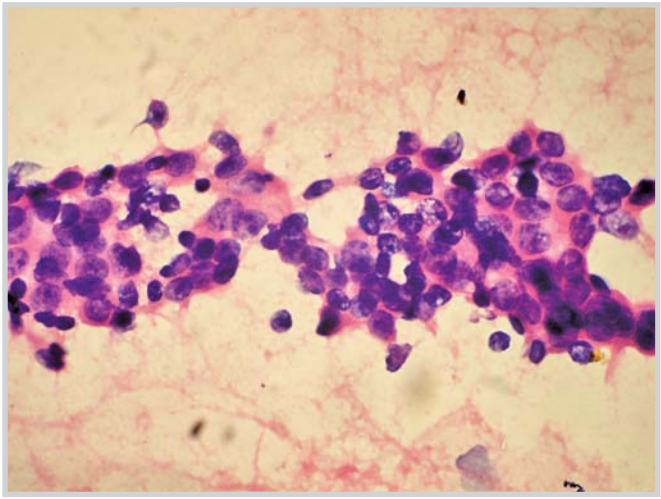

Fig. 27.21: Cluster of cells showing nuclear pleomorphism and irregular clumped chromatin in carcinoma in situ (H & E × MP)

material. Individual cells show moderate nuclear enlargement and pleomorphism along with mitotic activity. Individual cell morphology of comedocarcinoma and IDC is not different. Therefore, to distinguish these two entities in FNAC smear is a controversial issue. Lilleng and Hagmar suggested that cohesive tumor cells in the background of necrotic cellular debris favors DCIS of comedo type whereas, numerous dissociated tumor cells along with occasional clusters of cells in a background of minimal or absent necrosis indicates IDC.[43] FNAC smears of noncomedo type of DCIS show solid, papillary and cribriform clusters of cells (**Box 27.14**). The individual cells are small to intermediate in size and monomorphic in appearance. The cells look oval to polygonal with round nuclei and small nucleoli. Myoepithelial cells are absent in the smear. In addition foamy histiocytes, apocrine cells and calcified material may also be seen in the background.[44] In FNAC smear, the noncomedo type of DCIS is difficult to categorize exactly and the lesion may be labeled as suspicious for malignancy.

Differential Diagnosis

- Atypical ductal hyperplasia versus ductal carcinoma in situ: Differentiating ADH from DCIS is difficult. Smear of ADH is usually moderately cellular. The epithelial cells are arranged in cribriform and micropapillary pattern along with admixture of benign monolayered fragments. The myoepithelial cells are also present in ADH that are absent in DCIS. In comedo type of DCIS, the background necrotic debris and calcification are helpful in diagnosis. However at time, noncomedo type DCIS is difficult to distinguish from ADH as both lesions may display similar cytomorphologic features.

Phyllodes Tumor[45]

Phyllodes tumor (PT) represents about 0.3–1% of all primary tumors of breast. The older terminology "cystosarcoma phyllodes" is inappropriate because it gives a wrong impression about malignant nature of the tumor whereas the majority of PTs are benign. This tumor occurs in middle aged women and the patients are about 20 years older than the age group of FAd. The patient usually presents as a slow growing solitary well-circumscribed mass in the breast and the average size is about 5 cm at the time of presentation. The tumor may often be mistaken clinically as carcinoma due to the massive size of PT. Recurrence of PT is common and about 15–20% tumors recur after excision. On histology, PTs are classified as benign, borderline and malignant type.

Cytology (Figs 27.22 and 27.23A to C)

Cytology of the PT is more like FAd and the smear shows a biphasic cellular tumor composed of both stromal and epithelial cells with relative abundance of stromal component (**Box 27.15**). There are abundant spindle-shaped cells embedded in the fibromyxoid stroma. The spindle cells with plump fusiform nuclei

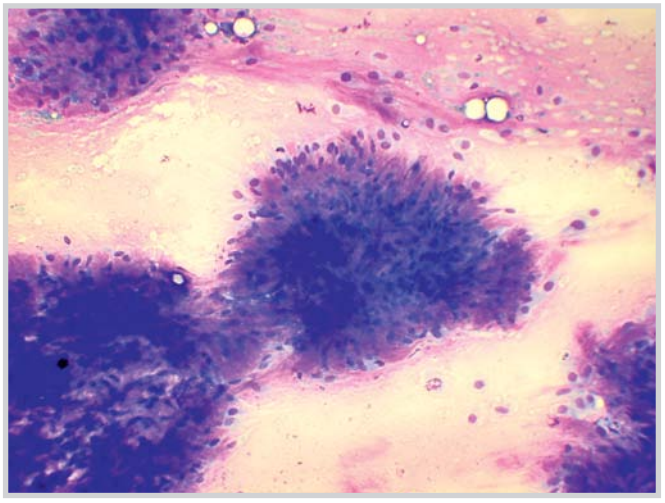

Fig. 27.22: Phyllodes tumor: multiple clusters and discrete oval to spindle-shaped stromal cells embedded in abundant pinkish stroma (MGG × MP)

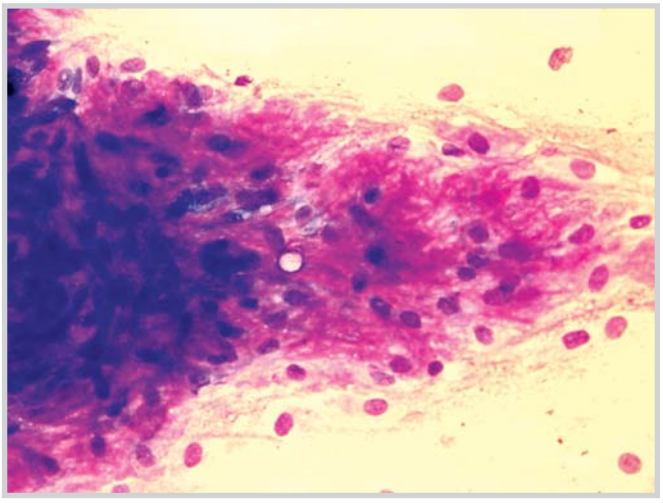

Fig. 27.23B: Phyllodes tumor: higher magnification of the same (MGG × HP)

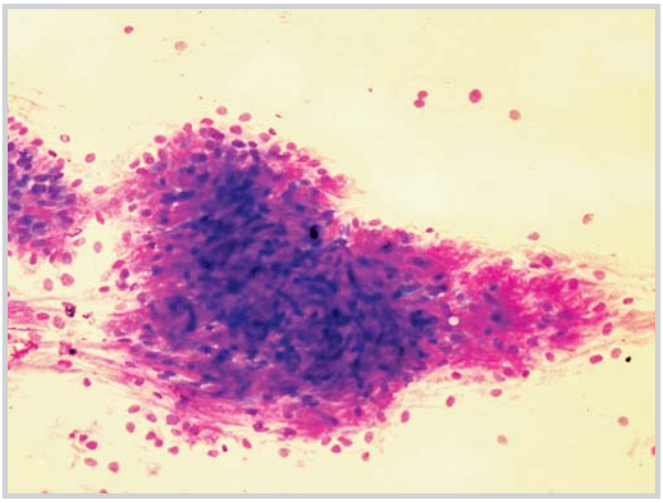

Fig. 27.23A: Phyllodes tumor: large clusters of spindle cells embedded in deep magenta colored material (MGG × MP)

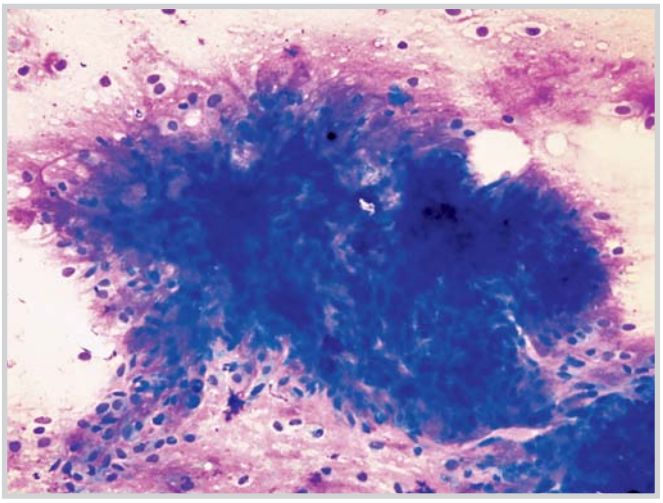

Fig. 27.23C: Phyllodes tumor: individual cells are oval to spindle-shaped with blunt ended nuclei (MGG × HP)

BOX 27.15 Phyllodes tumor

- Biphasic cellular tumor
- Both stromal and epithelial cells
- Abundant stromal component
- Abundant plump spindle-shaped cells
- Large folded sheets of benign monomorphic ductal cells

are also present discretely. The nuclei are usually monomorphic, however, mild degree of atypia may also be noted. In addition, there are multiple large folded sheets of benign monomorphic ductal cells. The smear may also show apocrine cells, foam cells and multinucleated giant cells. Benign PT does not show increased mitotic activity or any necrosis. Occasionally, the needle may pick-up the myxoid stromal material only and the smear may show low cellularity. This hypocellular smear of PT is difficult to diagnose. Therefore, a thorough sampling is always needed. Occasionally, PT may show predominantly epithelial hyperplasia with nuclear pleomorphism. Selective sampling of these areas may lead to a false-positive diagnosis of carcinoma of breast.[30]

Malignant PT shows large fragment of atypical stromal cells with significant nuclear pleomorphism that often simulates fibrosarcoma (**Figs 27.24** and **27.25**). In addition, the smear may also show high mitotic activity and necrosis.[46-48]

The stromal overgrowth in malignant PT may be so significant that there may be almost complete absence of epithelial cells and this may make difficult to distinguish these lesions from the pure sarcoma. However, pure sarcoma such as dermatofibrosarcomas and malignant fibrous histiocytomas are exceedingly rare in breast.[49]

Differential Diagnosis

- Fibroadnoma versus phyllodes tumor: PT shows relative abundance of stromal component over the epithelial component.

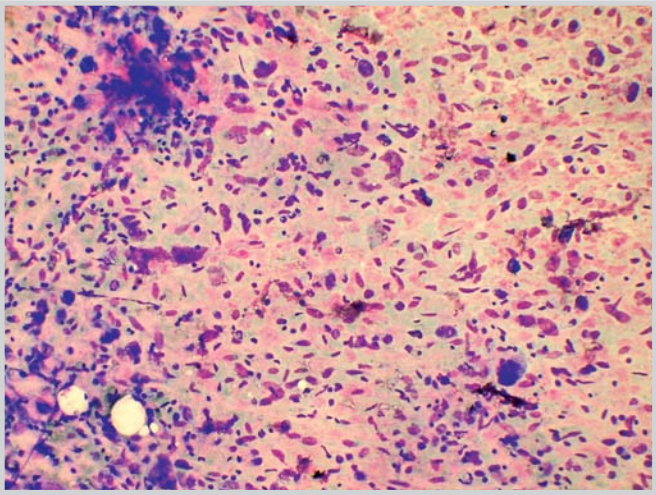

Fig. 27.24: Abundant discrete spindle cells with moderate nuclear pleomorphism in a case of malignant phyllodes tumor (MGG × MP)

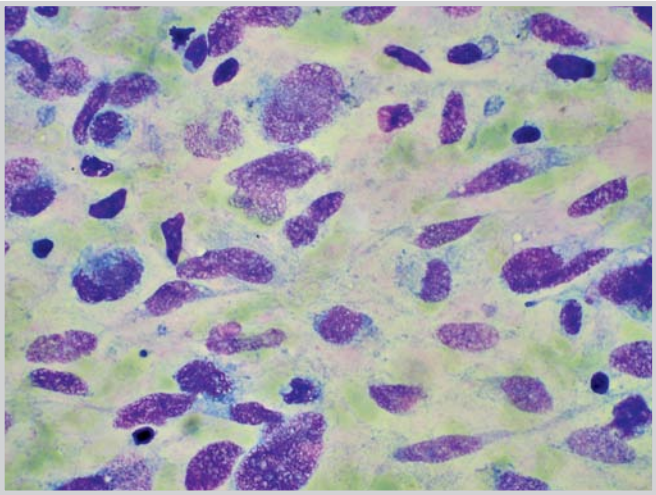

Fig. 27.25: Higher magnification showing large oval to spindle cells with markedly pleomorphic nuclei and irregular coarse chromatin in malignant phyllodes tumor (MGG × HP)

- Infiltrating duct carcinoma
- Metaplastic carcinoma: Here, the epithelial and stromal, both components are malignant. A careful observation of the benign epithelial component of PT may resolve the issue.

Papillary Neoplasm

Papilloma is a benign neoplasm characterized by the proliferation of the epithelial cells around the fibrovascular stalks. Recognizable myoepithelial cells are present in benign intraductal papilloma. It may arise from anywhere in the duct of the breast and most commonly seen near the areola. Papilloma mostly occurs in older woman; however, it may also affect the younger female. The tumor is usually solitary and obstructs the duct causing blood-stained nipple discharge. Cytological evaluation of the nipple discharge is usually the method of detection of papilloma and FNAC is uncommonly needed as the lesion is small and subareolar in majority of times.

Papillary carcinoma accounts for 1–2% of all breast carcinomas. This tumor may be cystic or solid and invasive or noninvasive. The overall prognosis of papillary carcinoma is better than IDC. In case of intraductal papillary carcinoma, the fibrovascular stalks are lined by epithelial cells and are almost totally (more than 90%) devoid of any myoepithelial cells. The important histological features of papillary carcinoma are:

- Absence of myoepithelial cells around the fibrovascular core
- Absence of apocrine cells
- Monomorphic cells with hyperchromatic nuclei
- Positive for smooth muscle actin (SMA) and high molecular weight cytokeratin (HMW-CK), the markers of myoepithelial cells.

Cytology[49] (Figs 27.26 to 27.28A and B)

The cytology smears are usually hypercellular and shows multiple papillary structures with fibrovascular core (**Box 27.16**). Smaller fragments of the tip of the papillae may also be seen. The ductal cells are arranged as palisading manner at the edge of the papillae. The individual cells are cuboidal to columnar with round nuclei and fine granular chromatin. The nuclei may show varying degree of nuclear enlargement and pleomorphism. Naked bipolar myoepithelial cells are seen. In addition, the background shows hemosiderin laden macrophages and apocrine cells. In FNAC, it is almost impossible to differentiate an intraductal papilloma from a papillary carcinoma. Therefore, it is better to label it as "papillary lesion" only. However, certain cytological features such as monomorphic nuclei, nuclear hyperchromasia, abundant discrete columnar cells, complete absence of myoepithelial cells and hemorrhagic background are the indicators of papillary carcinoma (**Box 27.17**).

Differential Diagnosis

- Fibroadenoma
- Papillary carcinoma versus papilloma.

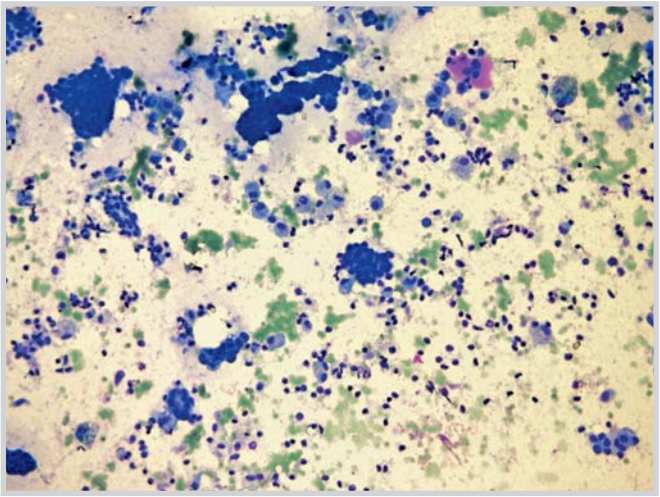

Fig. 27.26: Small papillae-like structures and many macrophages in papillary neoplasm of breast (MGG × MP)

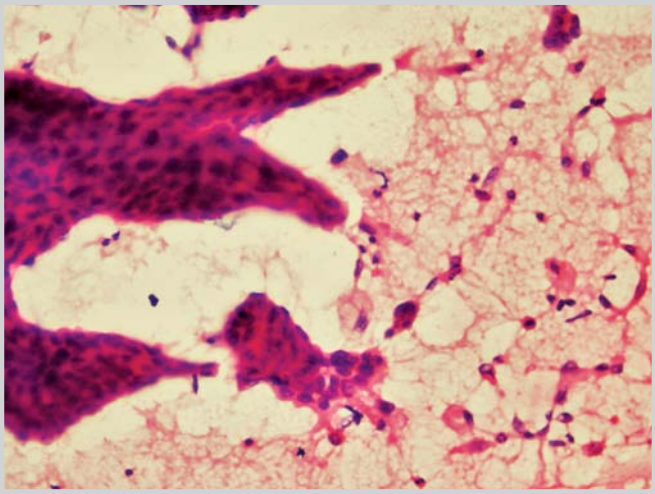

Fig. 27.27: Papillary neoplasm of breast: papillary structure along with many foamy macrophages and myoepithelial cells (H & E × HP)

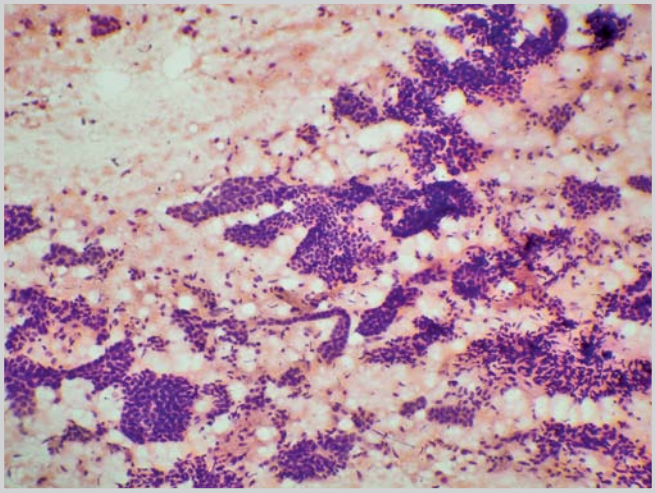

Fig. 27.28A: Abundant avascular papillary structures and occasional bipolar myoepithelial cells in papillary neoplasm of breast (H & E × MP)

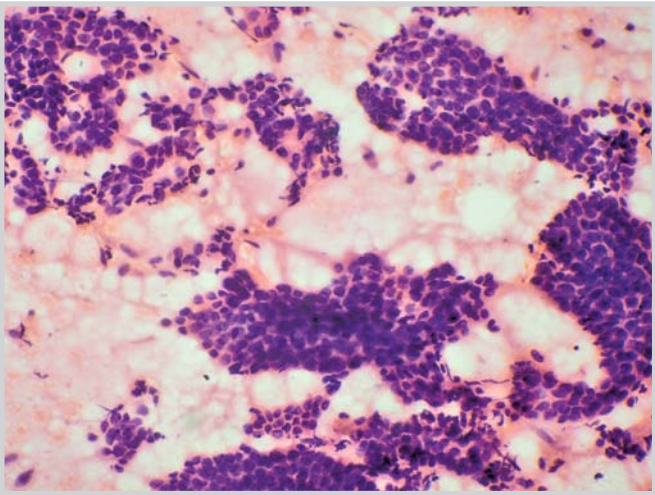

Fig. 27.28B: Higher magnification showing better morphology in papillary neoplasm of breast. Note the sharp margin of the clusters of cells. Individual cells are round with mildly pleomorphic nuclei (H & E × HP)

BOX 27.16 Papillary tumor of the breast

- Multiple papillae-like clusters of ductular cells with central stromal material
- Cuboidal to columnar cells arranged in regular manner at the edge of papillae
- Occasional discrete cell with intact cytoplasm
- Background shows foamy macrophages
- Varying amount of naked bipolar cells
- Varying degrees of nuclear atypia

BOX 27.17 Cytology features suggestive of papillary carcinoma

- Monomorphic nuclei
- Nuclear hyperchromasia
- Abundant discrete columnar cells
- Complete absence of myoepithelial cells
- Hemorrhagic background

Carcinoma

Infiltrating Duct Carcinoma

Infiltrating ductal carcinoma, not otherwise specified is the most common malignant tumor of the breast and represents about 70–80% of all breast cancers. IDC is a heterogenous group of tumor and lack specific or characteristic histologic pattern. It occurs predominantly in older women and is uncommon in the female younger than 35 years of age. The risk factors of the breast carcinoma include early menarche, late menopause, high intake of saturated fat in diet, nulliparity and history of first degree relative with breast cancer. Most of the IDC have characteristic mammographic pattern such as spiculated mass with microcalcification. USG of the IDC shows hypoechoic mass with altered echotexture and ill-defined border. The patient usually presents with hard fixed palpable mass. The overlying skin may be ulcerated and nipple may show retraction.

Cytology (Figs 27.29 to 27.32)

FNAC smears of IDC are highly cellular. The smears show multiple clusters and dissociated ductal cells with nuclear overlapping or crowding (**Box 27.18**). The ductal cells have enlarged pleomorphic and hyperchromatic nuclei with thickened and irregular membrane. Nucleoli are multiple and prominent. The chromatin shows irregular clumping. Smears show the complete absence of naked bipolar cells. It is important to give attention on the single isolated malignant cells. Without their presence the diagnosis of malignancy should be given with caution. Similarly, the presence of bipolar cells should be taken as serious evidence against the malignant nature of the tumor. At time, it may be difficult to distinguish a low-grade ductal carcinoma versus proliferative breast lesion with atypia. Granular to vesicular nuclear chromatin and thickened irregular nuclear membrane are useful cytological features of low-grade carcinoma.

About 70–80% of breast carcinomas are positive for estrogen receptor (ER) and 15–30% cases are positive for human epidermal growth factor receptor 2 (HER2-neu).

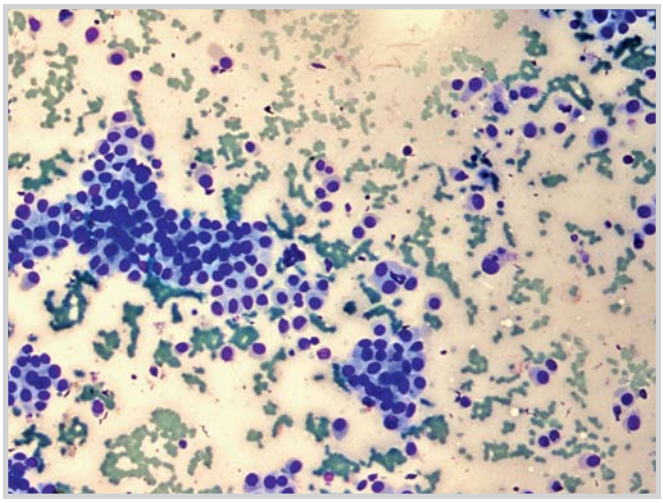

Fig. 27.29A: Loose clusters and discrete malignant cells in infiltrating duct carcinoma of breast (MGG × MP)

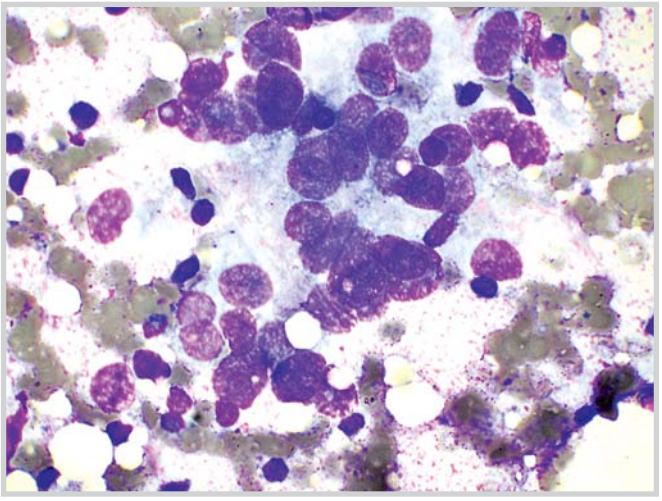

Fig. 27.30: Malignant cells with gland formation in infiltrating duct carcinoma of breast (MGG × OI)

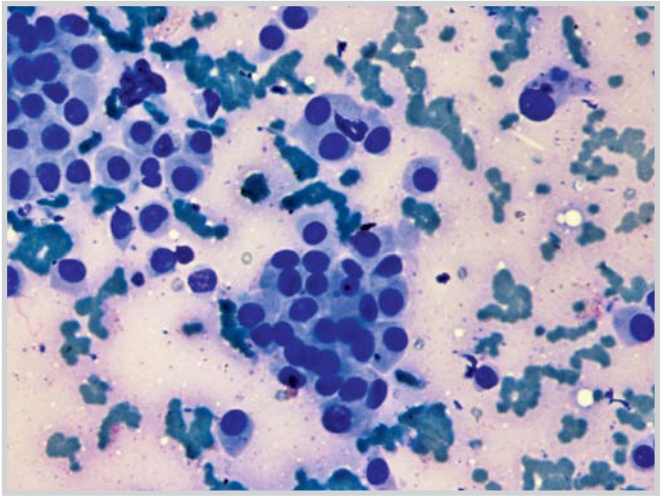

Fig. 27.29B: Higher magnification of the same showing better cell morphology (MGG × HP)

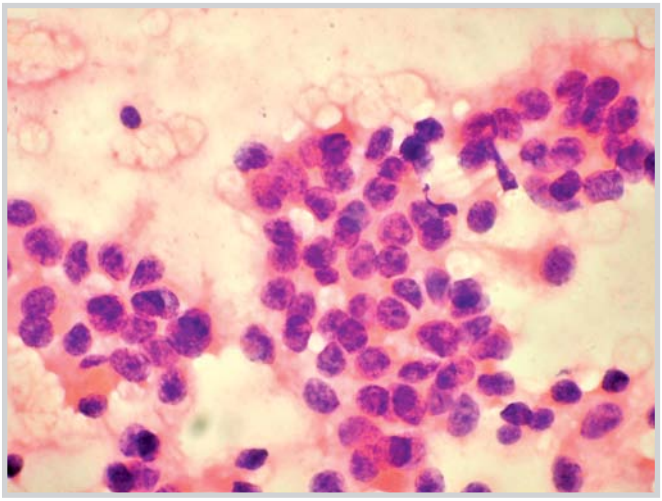

Fig. 27.31: Malignant cells showing irregular clumped chromatin in infiltrating duct carcinoma of breast (H & E × HP)

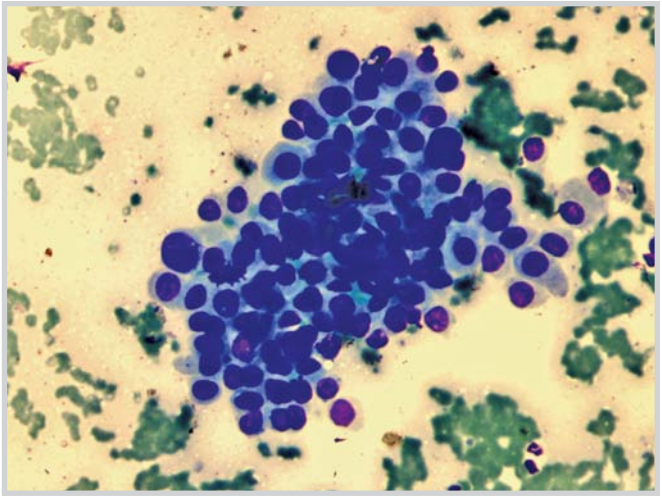

Fig. 27.29C: Cluster of malignant cells with overlapping pleomorphic nuclei. Occasional mitosis is seen within the cluster (MGG × HP)

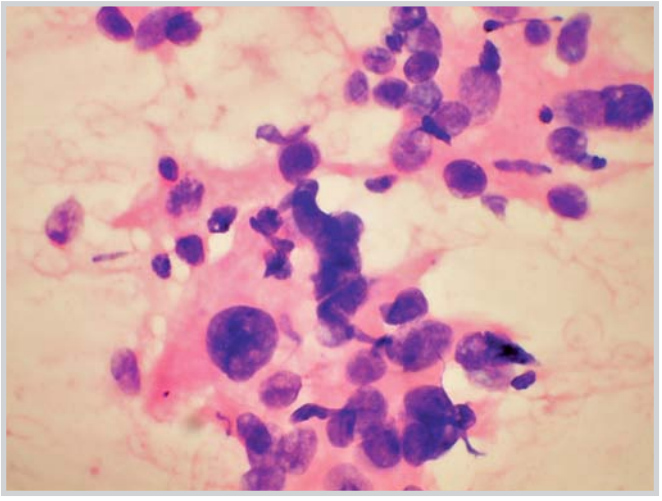

Fig. 27.32: Higher magnification showing nuclear margin irregularity, prominent nucleoli and clumped chromatin (H & E × OI)

BOX 27.18 Infiltrating duct carcinoma

- Hypercellular
- Clusters and dissociated ductal cells
- Nuclear crowding
- Often eccentric nucleus protruding out from the cytoplasm giving a plasmacytoid appearance
- Enlarged, pleomorphic, hyperchromatic nuclei
- Nuclear margin thickened and irregular
- Fine to coarse clumped chromatin and prominent nucleoli
- Myoepithelial cell absent
- Tumor diathesis

BOX 27.19 Medullary carcinoma

- Abundant dissociated cells
- Syncytial clusters of malignant cells
- Large cells with moderate nuclear pleomorphism
- Multiple prominent nucleoli
- Naked nuclei with macronucleoli
- Lymphocytes and plasma cells
- Tumor necrosis

Diagnostic Difficulties

- Low-grade carcinoma versus proliferative breast lesion with atypia
- Usual ductal carcinoma of low grade versus lobular carcinoma
- Pregnancy and lactational changes.

Other Types

Medullary Carcinoma

Medullary carcinoma accounts for 1–7% of all breast carcinomas. It is more common in the younger females. Clinically, the tumor may simulate as a FAds or cyst in the breast. The patient presents as well-circumscribed, solitary, soft and mobile mass. As this carcinoma is well-circumscribed so it may be mistaken as FAd. It is important to identify this subtype of breast carcinoma because the tumor simulates the cytological features of high-grade carcinoma; however, the prognosis of medullary carcinoma is far better than high-grade IDC. The cytologic features are[51] described in **Box 27.19**.

Cytology (Figs 27.33 and 27.34)

Medullary carcinoma of the breast shows usually abundant dissociated malignant cells (**Box 27.19**). The cells are often seen as syncytial clusters. The tumor cells are large, undifferentiated with moderately pleomorphic nuclei having large prominent nucleoli. Many malignant naked nuclei are present. Background of the smear shows moderate lymphocytes and plasma cell infiltration. This is an important diagnostic feature of medullary carcinoma of breast. At times, the presence of the bare nuclei may simulate pregnancy and lactational induced changes in the breast and vice versa. The presence of large amount lymphoid cells in the aspirates of medullary carcinoma may be confused with metastatic carcinoma in the intramammary lymph node. This tumor often lacks ER or progesterone receptor (PR) and HER2-neu expression.

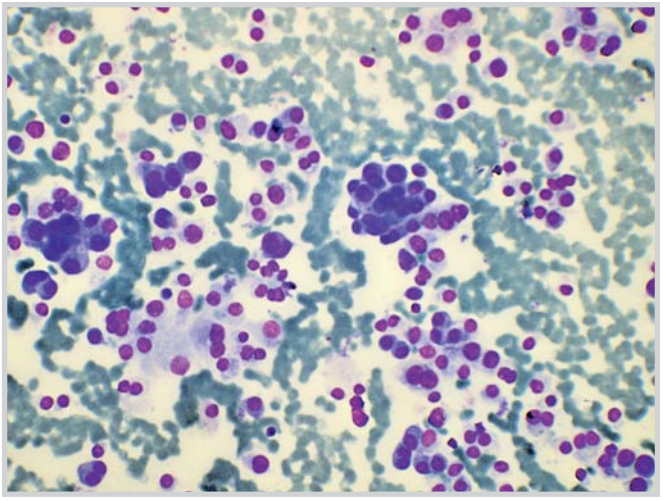

Fig. 27.33: Predominantly discrete cells in medullary carcinoma of breast (MGG × MP)

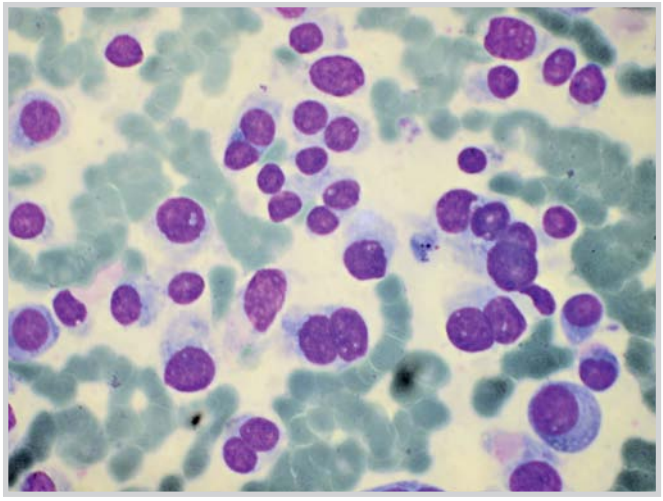

Fig. 27.34: Higher magnification showing pleomorphic nuclei and prominent nucleoli in medullary carcinoma of breast (MGG × HP)

Differential Diagnosis

- Poorly differentiated high-grade ductal carcinoma
- Metastatic breast carcinoma in an intramammary lymph node
- Chronic mastitis
- Lymphoma.

Mucinous Carcinoma (Colloid Carcinoma)[52]

Mucinous carcinoma is also labeled as colloid, mucoid or gelatinous carcinoma. Pure mucinous carcinoma represents 2% of breast carcinomas. The tumor occurs in all age groups;

however, the mean age of occurrence of mucinous carcinoma is higher than that of IDC. It is important to diagnose mucinous carcinoma because the tumor carries an excellent prognosis with 10-year survival in excess of 80%.[53] The patient presents as large slowly growing well-circumscribed lump in the breast. On mammography, the tumor may appear as a benign neoplasm.

Cytology (Figs 27.35 to 27.37)

FNAC of mucinous carcinoma yields thick mucoid material. The smears show abundant pale blue acellular mucinous material (**Box 27.20**). The loose clusters and discrete malignant cells float in the pool of mucin. The cells are blunt looking and may be mistaken as benign cells. Individual cells are monomorphic with moderate to abundant cytoplasm and round monomorphic nuclei. The chromatin of the nuclei is bland. Nucleoli are usually absent. Occasional cells show moderate nuclear enlargement and pleomorphic. Thin endothelial-lined vessels within the mucin are also described in mucinous carcinoma.[52] Mucinous material may also be noted in IDC, not otherwise specified. However, the amount of mucinous material is less in that case and cells show more nuclear atypia.

Mucinous material may be aspirated in different benign and malignant lesions of the breast and this should be remembered before the diagnosis of mucinous carcinoma (**Box 27.21**).

Differential Diagnosis

- Mucocele of the breast: Mucocele of the breast lesion may also yield abundant mucoid material. Mucocele like lesions of the breast usually show sheets of benign ductular cells with scanty cytoplasm and monomorphic nuclei.[54] In addition, background may show histiocytes and apocrine cells (**Table 27.5**).
- Myxoid change in fibroadenoma: Myxoid change in FAd may be confused with colloid carcinoma. FAd is usually cellular and show large tight clusters of cells along with many bipolar

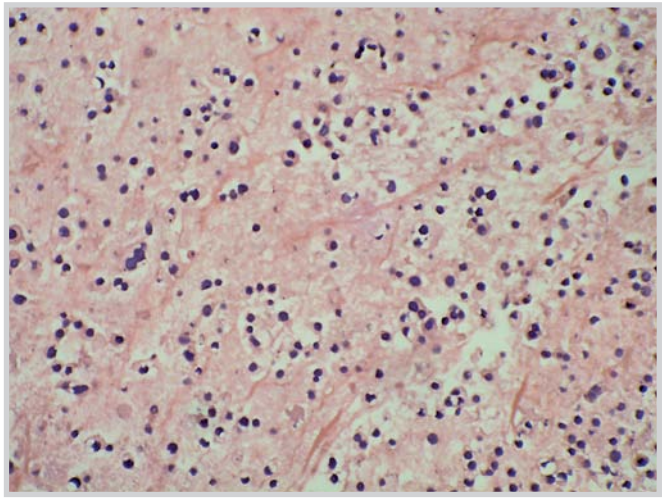

Fig. 27.35: Cell block preparation of medullary carcinoma of breast showing discrete cells admixed with lymphocytes (H & E × MP)

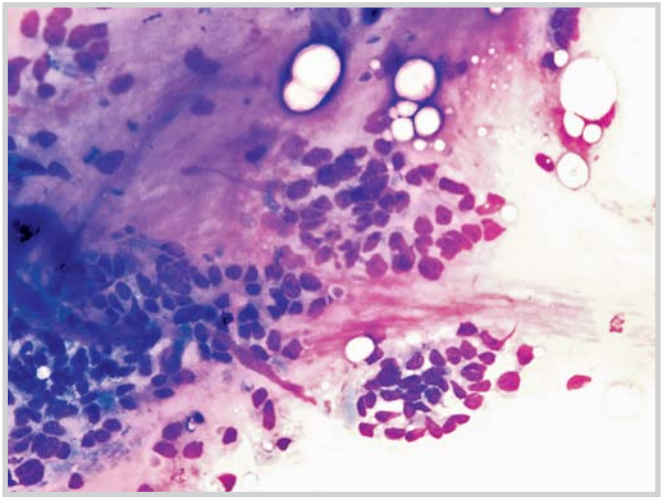

Fig. 27.37: Higher magnification showing round to oval pleomorphic cells in reddish bluish material (MGG × MP)

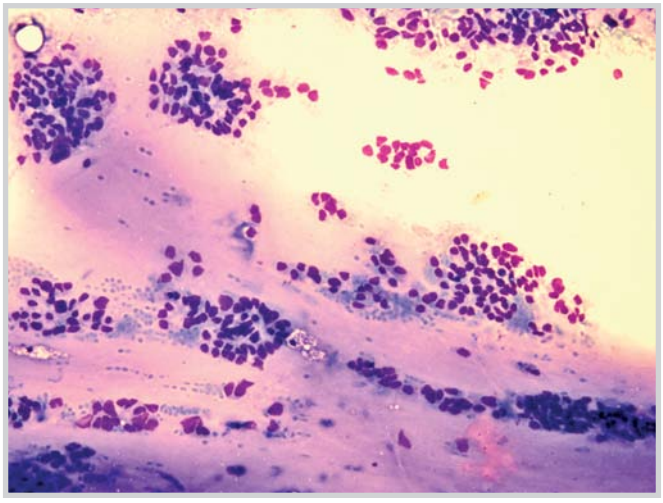

Fig. 27.36: Malignant cells embedded within the pool of mucin in mucinous carcinoma of breast (MGG × MP)

BOX 27.20 Mucinous carcinoma

- Abundant mucinous material
- Floating malignant cells in the pool of mucin
- Cells with abundant cytoplasm and mild to moderately pleomorphic nuclei
- Bland nuclear chromatin

BOX 27.21 Mucinous material in breast fine needle aspiration cytology (FNAC)

- Mucocele
- Mucinous adenocarcinoma of breast
- Mixed mucinous and ductal carcinoma
- Mucinous papillary neoplasm
- Secretory carcinoma

TABLE 27.5: Distinguishing features between mucinous carcinoma and mucocele

Features	*Mucinous carcinoma*	*Mucocele*
Cellularity	Mild to moderate	Scanty
Cell arrangement	Loose clusters and discrete	Discrete
Nuclear atypia	Mild to moderate	Nil
Background cell	Thin wire-like capillaries	Macrophages, apocrine cells and benign ductular cells

cells in the background. If it is not possible to distinguish myxoid FAd from colloid carcinoma then the tumor should be excised for histopathology.[52]

- Ductal carcinoma with mucinous metaplasia.

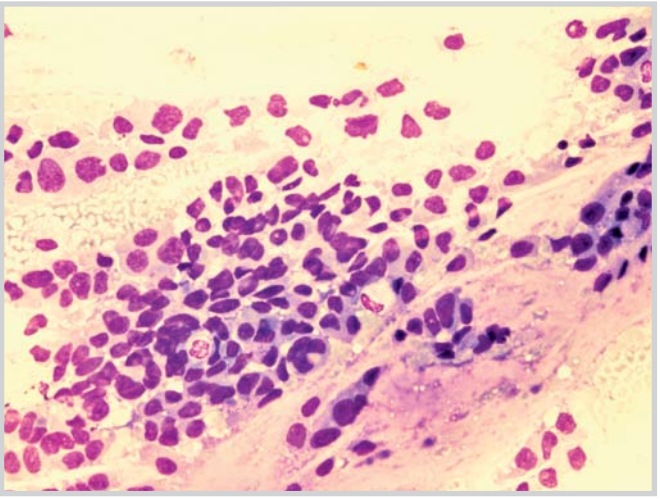

Fig. 27.38: Higher magnification showing cells with moderate nuclear enlargement and pleomorphism. The malignant nature of the cell is important in diagnosis of mucinous carcinoma (MGG × HP)

Apocrine Carcinoma

Apocrine carcinoma is a relatively uncommon malignancy and represents 0.3–4% of all breast cancers. This tumor is characterized by a malignant tumor with predominant population of apocrine cells (more than 90%). It usually affects the women of older age group. There is no special clinical feature of apocrine carcinoma to distinguish it from IDC.

Cytology (Figs 27.39 and 27.40)

Cytology smear of apocrine carcinoma is cellular. The cells are predominantly arranged as sheets, cords, tubules and discretely (**Box 27.22**). The individual cells are morphologically recognizable as apocrine cells. The cells are large with abundant granular eosinophilic cytoplasm. The nuclei are centrally placed large and pleomorphic having prominent nucleoli. Frequent binucleation and multinucleation are also noted. At times, the nuclei are eccentric in position and give a comet like appearance. Background necrosis may also be seen.

Apocrine change is common in FD and it usually indicates benign nature of the lesion. Mild degree of nuclear enlargement is also common in apocrine cells. So, cytologist should be careful to report an apocrine carcinoma. In fact, apocrine carcinoma is the gray zone tumor and often be mistaken as benign apocrine changes or vice versa (**Table 27.6**).

It is important to remember that apocrine changes are noted in IDC. Therefore, mere presence of apocrine cells may not indicate apocrine carcinoma and significant amount of apocrine cells is needed to diagnose apocrine carcinoma.

Differential Diagnosis

- Apocrine metaplasia
- Apocrine changes in other carcinomas.

Tubular Carcinoma

Tubular carcinoma represents l–2% of all breast carcinomas. This is a special type of breast carcinoma with well-differentiated

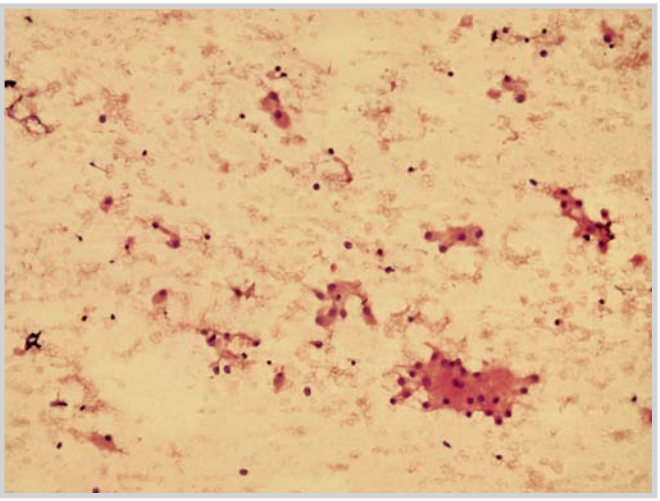

Fig. 27.39: Loose clusters and discrete apocrine cells in apocrine carcinoma of breast (H & E × MP)

BOX 27.22 **Apocrine carcinoma**

- Clusters and sheets of cells
- Abundant apocrine cells with moderate to abundant granular cytoplasm
- Centrally placed large vesicular nucleus
- Mild to moderate nuclear pleomorphism and prominent nucleoli
- Comet-shaped cells with protruding nuclei
- Immunostain: Positive for GCDFP-15, a marker of apocrine cells and negative for ER/PR

Abbreviations: GCDFP-15, gross cystic disease fluid protein-15; ER, estrogen receptor; PR, progesterone receptor

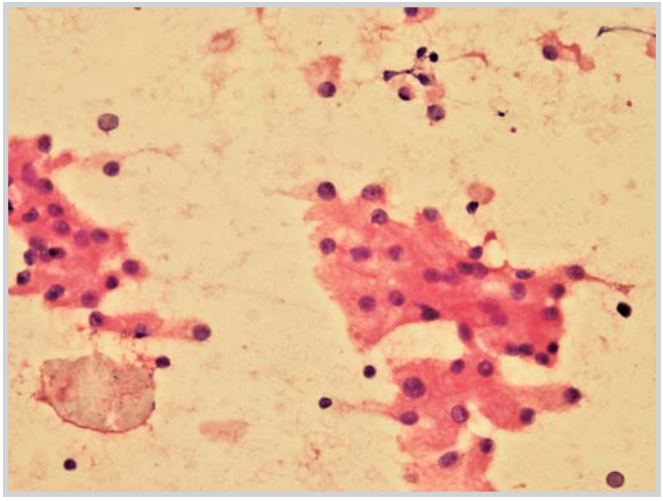

Fig. 27.40: The cells show mild nuclear enlargement and pleomorphism in apocrine carcinoma of breast (H & E × MP)

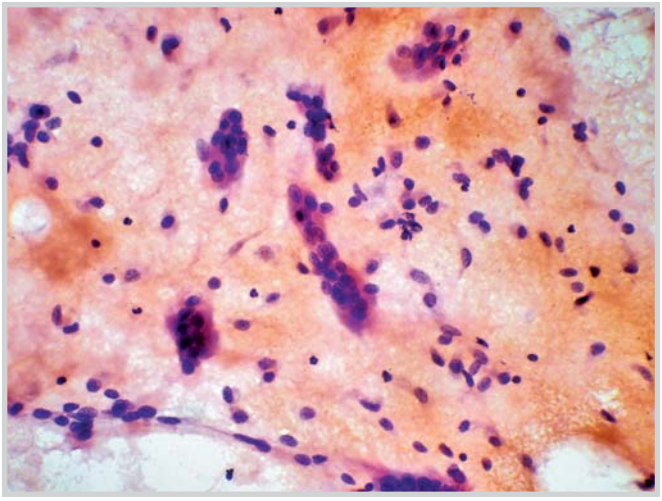

Fig. 27.41: Metaplastic carcinoma: many multinucleated giant cells and scattered oval to spindle cells (H & E × MP)

TABLE 27.6: Differentiating metaplastic apocrine cells and apocrine carcinoma

Features	*Benign metaplastic apocrine cells*	*Apocrine carcinoma*
Age	Younger	Older
Nuclei	Normal	Mild to moderate enlargement
Nucleoli	Single	Multiple prominent
Comet shaped cells	Absent	Present

BOX 27.23 **Tubular carcinoma**

- Usually scanty cellularity
- Small tubular structures
- Cohesive cells arranged in comma shaped manner
- The cells with monomorphic nuclei, finely granular chromatin and small nucleoli
- Absence of bipolar naked cells in the background
- Densely collagenized fibrous tissue fragments

tubular structures. Tubular carcinoma occurs in older patients compared to IDC and usually bilateral, smaller in size at the time of presentation with less incidence of lymph nodal involvement.

Cytology

Aspirates of tubular carcinoma of the breast usually show scanty cellularity due to stromal fibrosis. The smears show many small well-defined tubular structures and tight cohesive clusters of cells (**Box 27.23**). The tubular wall is lined by small round to oval cells. The long cord of cells with angular comma shaped division is seen. The individual cells have monomorphic nuclei with granular chromatin and small nucleoli. There is scanty to complete absence of bipolar naked cell in the background. At times, dense collagenized tissue fragments are also noted in the background. Scanty amount of cells with monomorphic nuclei may often give rise to a false-negative diagnosis of tubular carcinoma. However, the absence of bipolar naked cells should raise the suspicion of carcinoma.

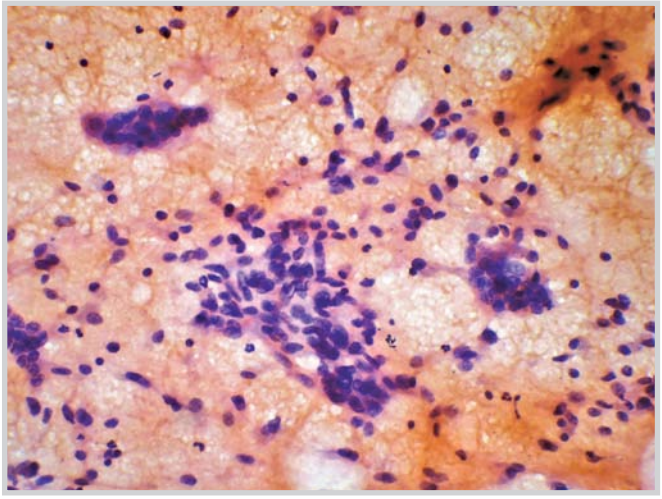

Fig. 27.42: Metaplastic carcinoma: multinucleated osteoclast like giant cells and malignant spindle cells (H & E × HP)

Diagnostic Difficulties

- Fibroadenoma
- Lobular carcinoma.

Metaplastic Carcinoma

This is a very uncommon malignancy of breast and accounts for less than 1% of breast carcinoma. This is a heterogeneous group of tumor characterized by adenocarcinoma along with dominant areas of spindle cell, squamous cells or mesenchymal differentiation. The average age of the patient is 55 years and clinical presentation is not different from IDC. This is an aggressive tumor with poor prognosis.

Cytology (Figs 27.41 and 27.42)

Cytology of this malignancy depends on the various component of the metaplastic carcinoma. The tumor may show adenocarcinoma with squamous cells. There may be prominent clusters of spindle cells, multinucleated tumor giant cells or malignant heterologous mesenchymal elements (**Box 27.24**).

BOX 27.24 **Cytology of metaplastic carcinoma**

- Epithelial cells with moderately pleomorphic nuclei
- Large clusters of spindle cells
- Multinucleated giant cells
- Squamous cells
- Malignant heterologous or homologous mesenchymal elements

BOX 27.25 **Signet ring carcinoma**

- Rich in cells
- Loose clusters and dissociated malignant cells
- Signet ring cells: Abundant cytoplasm and eccentric nucleus
- Positive for mucicarmine and periodic acid-Schiff (PAS) stain

Differential Diagnosis
- Sarcoma
- Cytosarcoma phylloides
- Fibromatosis.

Adenoid Cystic Carcinoma of Breast

This is an extremely uncommon tumor of breast and represents 0.1% of breast carcinomas. This tumor has very good prognosis. Cytology features of adenoid cystic carcinoma of breast are similar to that of breast. The smear shows many deep magenta colored globules surrounded by round to oval cells. Individual cells are small, round to oval with scanty cytoplasm and regular, hyperchromatic nuclei.

Differential Diagnosis
Benign collagenous spherulosis.

Signet Ring Carcinoma

This represents 2-4% of all breast carcinomas. The signet ring carcinoma usually occurs in higher age group. This is an aggressive carcinoma and more often involves the axillary lymph nodes.

Cytology

Fine needle aspiration cytology smears show loose clusters and dissociated malignant cells. Individual cells have abundant cytoplasm. The nucleus of the cell is pushed to the periphery and gives a signet ring like appearance (**Box 27.25**). Unlike mucinous carcinoma, this tumor show intracellular mucin. The mucin within the cell is positive for mucicarmine and periodic acid-Schiff (PAS) stain.

Invasive Lobular Carcinoma[55,56]

Invasive lobular carcinoma (ILC) accounts for 5–15% of all breast cancers.[56] ILC occurs in all age groups from third decade to nine decade. The lobular carcinomas are usually ill-defined and multicentric. At mammography, architectural distortion is commonly noted and microcalcification is less frequently seen.

Cytology (Figs 27.43 to 27.46)
The FNAC smears of lobular carcinoma of breast shows variable cellularity. There may be scanty cellularity due to stromal fibrosis.

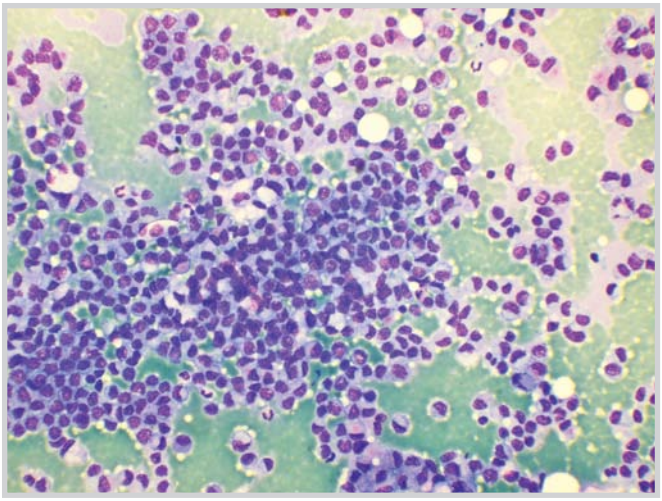

Fig. 27.43: Abundant discrete round mildly pleomorphic cells in lobular carcinoma of breast (MGG × MP)

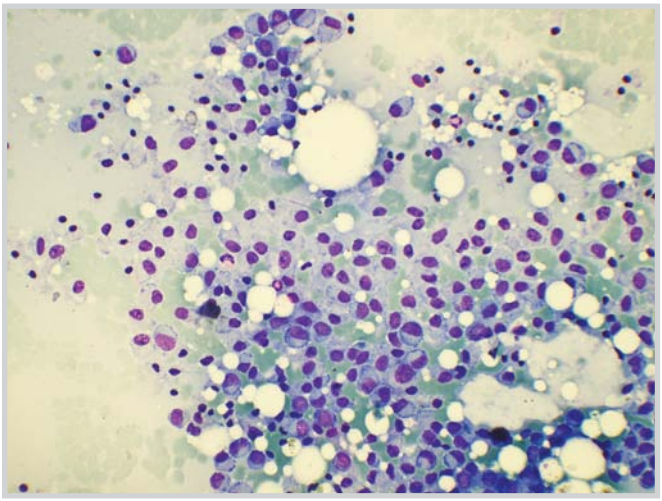

Fig. 27.44: Discrete cells with moderate cytoplasm having eccentric nuclei in lobular carcinoma of breast (MGG × MP)

The tumor cells are often arranged in cords, small clusters and discretely. The cells often are arranged in small rows giving rise to Indian file arrangement. The individual cells are small with scanty cytoplasm having hyperchromatic mildly pleomorphic nucleoli with inconspicuous nucleoli. Occasionally, the cells may compress with each other and may show nuclear molding.

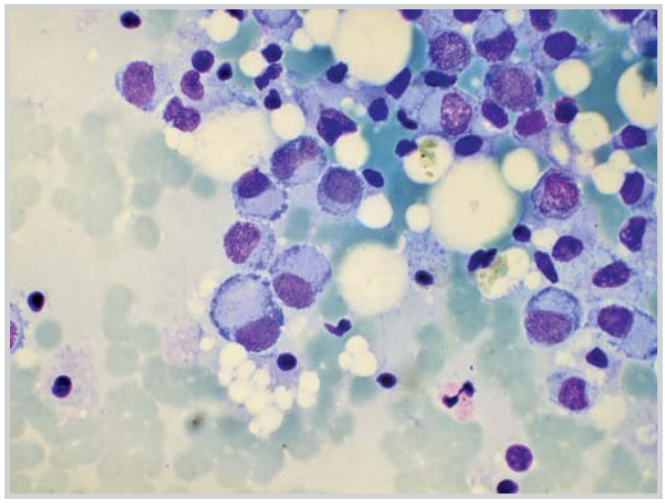

Fig. 27.45: Higher magnification showing cells with intracytoplasmic lumen in lobular carcinoma of breast (MGG × OI)

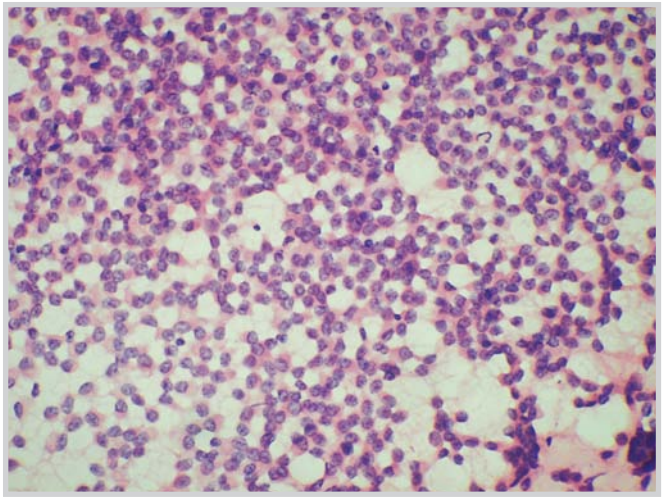

Fig. 27.46: Discrete monomorphic cells with bland nuclear chromatin in lobular carcinoma of breast (H & E × MP)

BOX 27.26 Lobular carcinoma

- Sparse to moderately rich cellularity
- Dissociated cells
- Small rows of cells known as Indian file arrangement
- Signet ring-like cells with small intracytoplasmic lumina
- Small cells with scanty cytoplasm having minimal nuclear pleomorphism
- Nuclear molding

Cytoplasm of the cells may be filled with mucin and nucleus may be pushed to the periphery giving rise to signet ring cells (**Box 27.26**). The intracytoplasmic lumen within the cell is one of the characteristics features of ILC.

TABLE 27.7: The differentiating points between lobular carcinoma and infiltrating duct carcinoma

	Lobular carcinoma	*Ductal carcinoma*
Indian file arrangement	Present	Absent
Cell size	Small sized cell	Relatively larger size
Nucleus	Monomorphic	Pleomorphic
Nuclear molding	Present	Absent
Intracytoplasmic lumina	Present	Absent
Signet ring cells	Present	Absent

BOX 27.27 Paget's disease

- Dissociated and occasional clusters of cells
- Large cells with clear cytoplasm and centrally placed large pleomorphic nuclei
- Inflammatory cells and squamous cells

Immunostain: Positive for CEA, EMA and negative for S-100 and Human melanoma black 45 (HMB-45).

Abbreviation: CEA, carcinoembryonic antigen; EMA, epithelial membrane antigen

Diagnostic Difficulties

- Benign lesion: Invasive lobular carcinoma shows lack of myoepithelial cells and may show several other characteristic cytological features.
- Ductal carcinoma: Monomorphic cells in ILC may simulate a low-grade IDC. However, the presence of Indian file like cells, nuclear molding and signet ring type of cells favor the diagnosis of lobular carcinoma. **Table 27.7** describes the differentiating points between lobular carcinoma and IDC.

Paget's Disease

Paget's disease typically presents as ulcerated eczematous lesion around the nipple and areola. In majority of the cases, an underlying breast carcinoma is also present.

Paget's disease is diagnosed by scraping of the ulcerated lesion.

Cytology

The cytology smears show dissociated and occasional clusters of large cells. Individual cells have clear cytoplasm and centrally placed moderately pleomorphic nuclei (**Box 27.27**). Occasional cell may contain melanin pigments that probably have been transferred from adjacent melanocytes. Background of the smears may show inflammatory cells and squamous cells. The presence of melanin pigment may mislead the cytologists to diagnose the lesion as malignant melanoma. Immunocytochemistry is helpful in this aspect as Paget's cells are positive for carcinoembryonic

antigen (CEA), epithelial membrane antigen (EMA) and ER, whereas, malignant melanomas are positive for HMB45 and S-100 protein.

Differential Diagnosis

- Malignant melanoma
- Inflammatory atypia: Nonspecific inflammation in areola or nipple may also produce reactive atypia. Nuclear features may be helpful in diagnosis of malignancy.

Inflammatory Carcinoma

This is an uncommon aggressive tumor and represents 2–4% of all breast carcinomas. Clinically the breast is erythematous, diffusely enlarged, edematous and tender. This is due to lymphatic obstruction by the tumor emboli. As there is no definite swelling so FNAC may not yield any representative material and aspiration from the multiple sites are recommended. Cytology smear shows discrete and clusters of large pleomorphic cells with prominent macronucleoli. Contrary to its name as "inflammatory carcinoma" no inflammatory cells are seen in the background.

Other Uncommon Malignancies

Carcinoma with Neuroendocrine Differentiation (Figs 27.47 and 27.48)

Neuroendocrine carcinomas are rare breast cancer and accounts for 2–5% of all breast cancers. Most patients are in six decade to seven decade of life. There is no special clinical feature of neuroendocrine carcinomas. These tumors are argyrophile but not argentaffin and show dense core granules. In true sense, these tumors are not carcinoid and they should be better designated as a type of IDC with neuroendocrine differentiation.

Cytology

The tumor shows abundant discrete cells. The cells are round and monomorphic with scanty to moderate cytoplasm. The nuclei are mildly pleomorphic with finely granular chromatin and inconspicuous nucleoli. The cells are positive for chromogranin and synaptophysin.

Metastatic Tumors (Fig. 27.49)

Breast is the uncommon site of the metastatic tumor and it represents 0.5–6% of all malignancies of the breast. The contralateral breast is the most common source of the metastasis in breast carcinoma. The other common sources of metastasis in adults are lung, kidney and gastrointestinal tract (GIT). In case of children, rhabdomyosarcoma is the most common metastatic tumors. In adult male, prostatic carcinoma is the common source of metastasis in male breast.

On the basis of cytology features, it is not always possible to distinguish a primary from a metastatic tumor of breast.

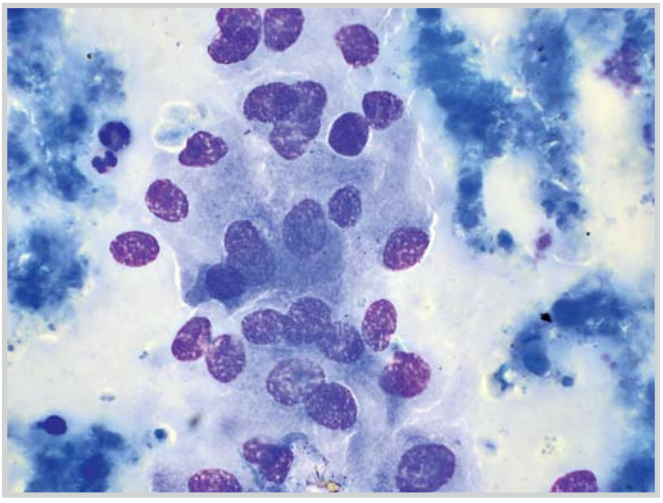

Fig. 27.48: Higher magnification showing better cell morphology of neuroendocrine carcinoma of breast. Nuclei of the cells show fine stippled chromatin and inconspicuous nucleoli (MGG × OI)

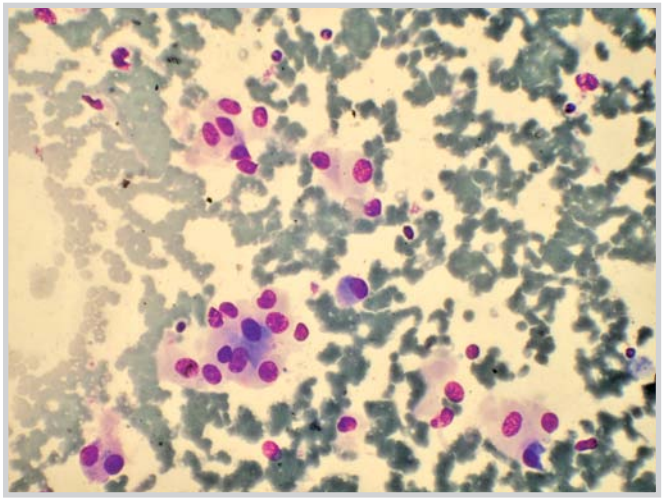

Fig. 27.47: Cytology smears of neuroendocrine carcinoma of breast showing discrete cells with moderate amount of cytoplasm and eccentric nuclei (MGG × MP)

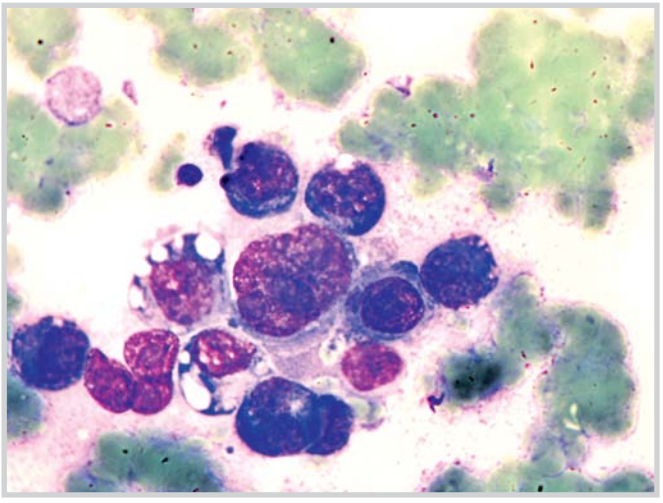

Fig. 27.49: Fine needle aspiration cytology (FNAC) smears of metastatic rhabdomyosarcoma in breast from a thigh swelling in a 7-year-old child (MGG × OI)

However, if the cytomorphology of the tumor does not fit with any known primary malignancy of the breast, then one should think about the possibility of a secondary tumor. Clinical history may be helpful in certain situations.

Non-Hodgkin Lymphoma

Non-Hodgkin lymphomas (NHL) of the breast, both primary and secondary, are rare. Most of the NHL affects the breast as a manifestation of generalized involvement. The clinical presentation of the primary NHL may mimic as breast cancer. The patient usually presents as a painless lump in the breast. In one-tenth of the cases the lump is bilateral. For the diagnosis of primary NHL of the breast, the following histological criteria are mandatory:[57]

- No prior history of lymphoma
- No nodal involvement of lymphoma at the time of presentation
- The presence of breast tissue in adjacent lymphoid tissue
- Histological confirmation of the case.

The common types of lymphomas in the breast are: (1) diffuse large B-cell lymphoma, (2) mucosa-associated lymphoid tissue (MALT) lymphoma and (3) follicular lymphoma.

The cytomorphology of NHL depends on the subtype of NHL.

Differential Diagnosis

- Chronic mastitis
- Intramammary lymph node.

Male Breast Lesions

Gynecomastia[58] (Figs 27.50 and 27.51)

The gynecomastia is the most common breast lesions in male and is caused by hypertrophy and hyperplasia of the ductal and stromal component of the breast. The main cause of gynecomastia is due to decreased androgenic hormone and increased estrogen. The gynecomastia is commonly associated with cirrhosis of liver, hyperthyroidism, renal failure, chronic lung disease, germ cell tumor of the testis and various drugs such as spironolactone, digitalis, cimetidine, marijuana and tricyclic antidepressants.

Cytology

The cytology smears of gynecomastia usually show sparse cellularity. The ductal cells are arranged in dissociation and occasionally in monolayer sheets. The individual cells show round nuclei with mild nuclear atypia. Discrete ductal cells do not show significant atypia. Nuclear crowding, significant dyscohesive cells and marked nuclear pleomorphism are the characteristic cytological features of malignancy. Bipolar myoepithelial cells are noted in the background.

Differential Diagnosis

- Ductal carcinoma: Nuclear atypia in gynecomastia may cause diagnostic difficulties with invasive duct carcinoma.

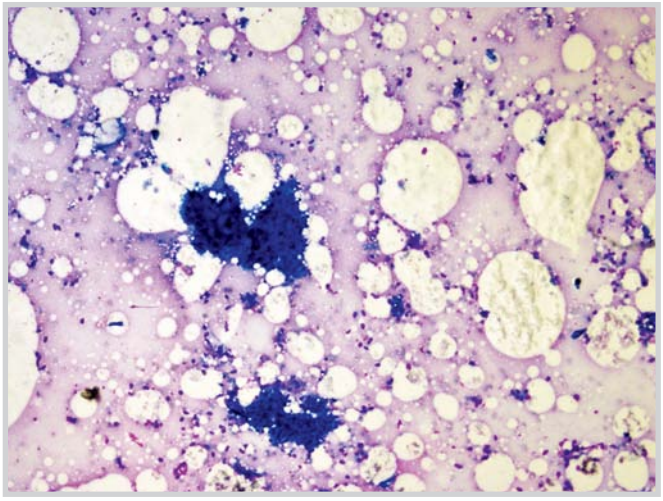

Fig. 27.50: Tight clusters of benign ductular cells in a case of gynecomastia (MGG × MP)

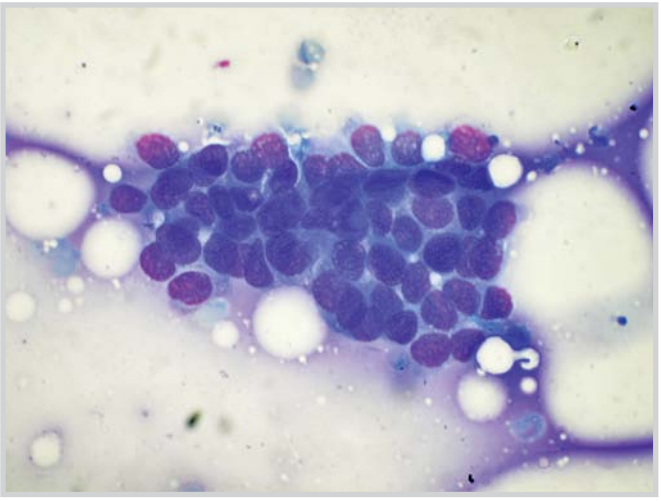

Fig. 27.51: Mild nuclear enlargement and pleomorphism in gynecomastia (MGG × HP)

Carcinoma in Male Breast (Figs 27.52 and 27.53)

Male breast carcinoma is a rare carcinoma and represents less than 1% of all carcinoma of the breast. It is more aggressive than female breast carcinoma. Almost all male breast carcinomas are ductal type. The cytomorphology of the male breast carcinoma is similar to that of female breast IDC. However, male breast carcinoma shows dyscohesive cells with marked nuclear enlargement and pleomorphism. Bipolar cells are absent in the smear.

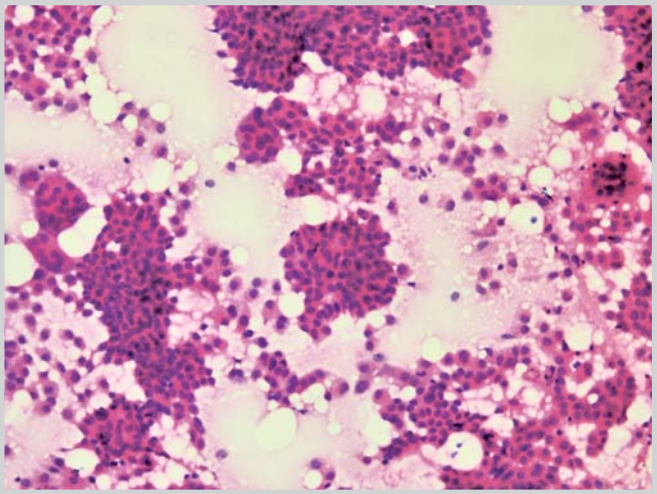

Fig. 27.52: Male breast carcinoma: abundant discrete malignant cells (H & E × MP)

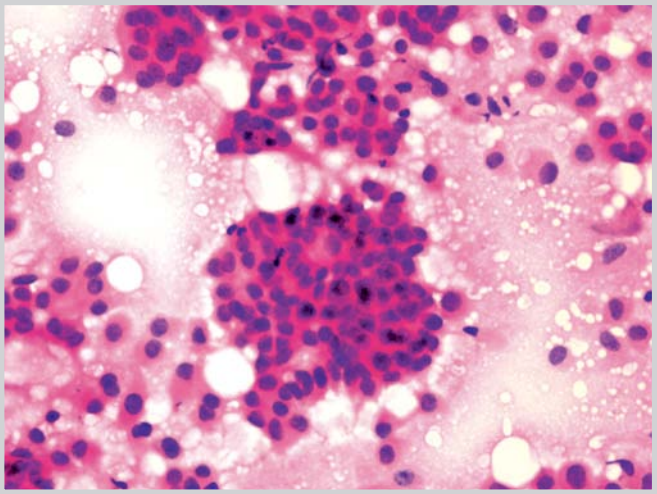

Fig. 27.53: Male breast carcinoma: higher magnification showing moderately pleomorphic cells with enlarged hyperchromatic nuclei (H & E × HP)

Ancillary Investigations on Breast Aspiration Material

Aspiration cytology material of the breast lesions can be used for various ancillary investigations such as immunocytochemistry, flow cytometry and microbial culture.

Immunocytochemistry

Direct smear, cytospin smear or section from the cell block could be used for immunocytochemistry of the breast lesions. Section from the cell block is preferable as it gives consistent and good result. Estrogen, progesterone and HER-2/neu receptors can be demonstrated on the aspirate material for the further management of carcinoma of breast.

Flow Cytometry and Image Cytometry

Information on deoxyribonucleic acid (DNA) ploidy and S-phase fraction can be assessed by flow cytometry and image cytometry.

REPORTING OF BREAST FINE NEEDLE ASPIRATION CYTOLOGY

In the symposium conducted by NCI of Bethesda, Maryland, United State of America (USA),[21] a uniform reporting system of the breast was proposed:

- *Benign*: No evidence of malignancy.
- *Atypical or indeterminate*: Mild atypia present, possibly benign lesion. However, clinical and radiologic correlation is advised. In this category, triple test is strongly recommended.
- *Suspicious or probably malignant*: Cytology smear shows many atypical cells and is highly suggestive of malignancy, however for a more definitive opinion histopathology should be advised.
- *Malignant*: Cytomorphological features are diagnostic of malignancy and further subcategorization should be mentioned, if possible.
- *Unsatisfactory*: The smear quality is substandard because of scant cellularity, air drying artifact, obscuration by blood or inflammation, etc.

National Cancer Institute sponsored symposium[21] also suggested that the cytology report should mention certain information in the reporting format such as:

Reporting format of FNAC of breast:

- *Precise location of the lesion*: Distance from the nipple and exact clock position
- *Type of specimen*: Whether solid or cystic lesion, scrapping, nipple discharge or FNAC material
- *Localization technique*: Whether palpable mass or image guided FNAC
- *Diagnostic terminology*: as discussed above
- *Adequacy of the aspirated material*: Adequate number of ductal cells, air drying artifact, etc.
- Comments on the specimen findings
- *Recommendations*: Comments on requirement of any other clinical and radiological correlations

Cytological Grade

Neoadjuvant therapy is now being applied as one of the treatment modalities of breast carcinoma and therefore grading of breast carcinoma is needed for the management. Histological grading of breast carcinoma is well-established. In FNAC material, cytological grading system has been also tried. Cytological grading by Robinson's et al. and Idvall I et al.[59,60] are well-correlated with histological grade. Robinson's et al. included six cytological criteria and each scored separately from one to three. Total score obtained in each sample is used for grading of breast cancer on FNAC smears (**Table 27.8**). In contrast, Idvall et al.[60] used an ocular micrometer to measure the nuclear diameter of the tumor cells. They used a total of six cytological features to grade the carcinoma. Both the grading systems can be applied

TABLE 27.8: Robinson's criteria for grading of breast cancer on fine needle aspiration cytology (FNAC) smears

Parameter	*Score 1*	*Score 2*	*Score 3*
Cell dissociation	Mostly in cluster	Mixture of single cell and cluster	Mostly single cell
Cell size	1–2 times of RBC	3–4 times of RBC	> 5 times of RBC
Cell uniformity	Monomorphic	Mildly pleomorphic	Pleomorphic
Nucleoli	Indistinct	Noticeable	Prominent
Nuclear margin	Smooth	Folds	Clefts
Chromatin	Vesicular	Granular	Clumped

Grade 1: 6–11 score, Grade 2: 12–14 score and Grade 3: 15–18 score
Abbreviations: RBC, red blood cell

on MGG or Papanicolaous' stained smear. However, the later stain is preferable to grade the breast carcinoma.

Ancillary Techniques

Ancillary investigations are helpful to provide some essential information on breast cancer. Following information is mandatory for management of breast carcinoma:

- Estrogen receptor and PR status
- HER-2/neu oncogene receptor status.

Prognostic parameters:

- Deoxyribonucleic acid ploidy and S-phase fraction
- Proliferation markers such as Ki 67 index.

Intracellular steroid hormone receptors particularly ER and PR have drawn considerable attention. Patients with hormone-receptor positivity, have higher 5 years survival rate.[61] Studies have shown that the hormone receptor–positive tumors are usually well-differentiated and diploid tumor with low proliferation rate. These tumors also show low propensity for visceral recurrence.[62,63]

Assessment of hormone receptors status is very essential for the preoperative management of breast carcinoma. Majority of ER or PR-positive cases are hormone responsive and only 1-10% of hormone receptor cases are hormone sensitive. The receptor status of breast carcinoma can be provided with the help of immunocytochemistry on cell block preparation (**Fig. 27.54**).[64] HER-2/neu oncogene amplification is one of the common genetic changes in breast carcinoma.[65] About 25–30% of breast cancer shows HER-2/neu oncogene over expression.

Carcinoma cases, which are HER-2/neu oncogene positive in fluorescence in situ hybridization (FISH) or by immunohistochemistry, show clinical response to trastuzumab (Herceptin) therapy. Both immunohistochemistry and FISH analysis can be done on FNAC sample.[66,67]

Over expression of HER-2/neu is demonstrated by immunocytochemistry using anti-HER-2/neu antibody. Similarly, HER-2/neu amplification is detected with the help of HER-2/neu DNA probe. Due to difficulty in the interpretation of membranous staining of HER-2/neu receptor, it is advisable to do FISH on FNAC material.

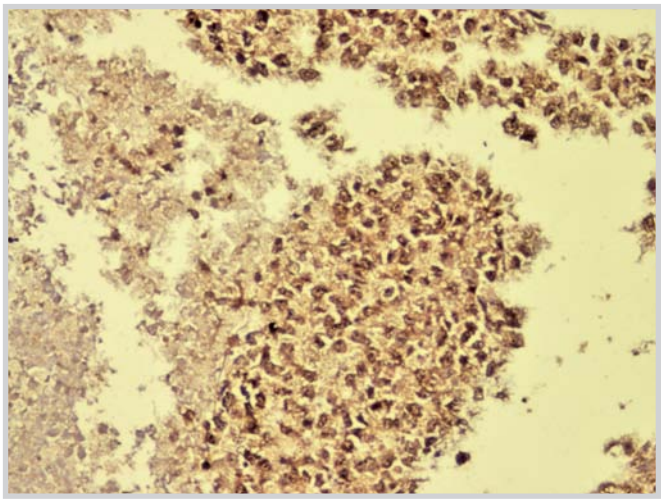

Fig. 27.54: Estrogen receptor immunostaining in cell block preparation of infiltrating ductal carcinoma of breast (MP)

Nipple Discharge

Nipple discharge may be due to physiological or pathological causes. Pregnancy and lactation are the physiologic causes of nipple discharge. The various pathological causes of nipple discharge are endocrine disorders such as hyperthyroidism and hypothyroidism, pituitary adenoma and drugs. The primary lesions of breast causing nipple discharges are intraductal papilloma, duct adenosis and ductal carcinoma. Only 3% of breast carcinoma presents nipple discharge and nipple discharge cytology is not suitable for cancer screening.[68] The nipple discharge is suspicious for malignancy when it is unilateral, bloody, associated with lump and mammography is abnormal. It is highly unlikely to have any malignant cells in milky or purulent nipple discharge.[69]

Cytology

Benign nipple discharge is usually sparsely cellular. Smear shows many foamy cells, occasional loose clusters of ductular cells, red blood cells (RBCs) and polymorphs (**Figs 27.55** and **27.56**).

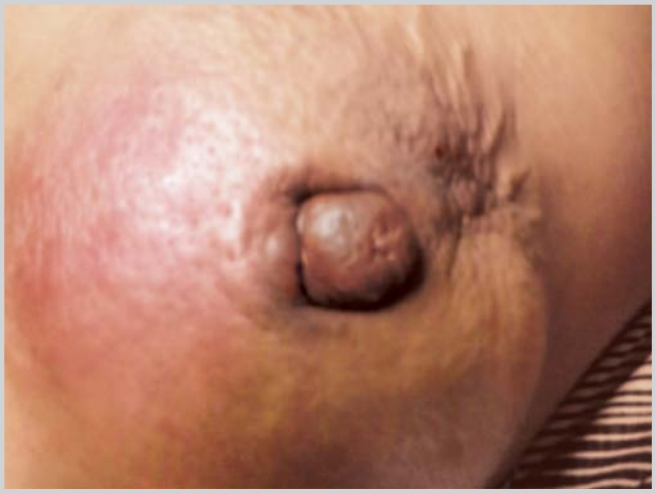

Fig. 27.55: Foamy histiocytes in nipple discharge smear (H & E × HP)

Cytology smear from the intraductal papilloma shows tight cohesive ductal cells often arranged as papillae. The lining cells of the papillae are columnar to cuboidal with scanty cytoplasm and monomorphic nuclei. In addition, foamy histiocytes are also noted. Exact nature of the papillary neoplasm is difficult to ascertain on cytology smear.

Ductal carcinoma in nipple discharge shows many discrete malignant cells (**Fig. 27.57**) (**Box 27.28**). Occasional cluster of cells with nuclear abnormality may also be seen. Degenerated ductal cells in nipple discharge may give false impression of malignancy. It is important to give adequate attention on the discrete ductular cells. All positive for malignancy in nipple discharge should have histopathological confirmation.

BOX 27.28 Ductal carcinoma in nipple discharge

- Discrete and loose clusters of cells
- Moderate nuclear enlargement and pleomorphism
- Naked nuclei
- Necrotic debris
- Polymorphs and blood

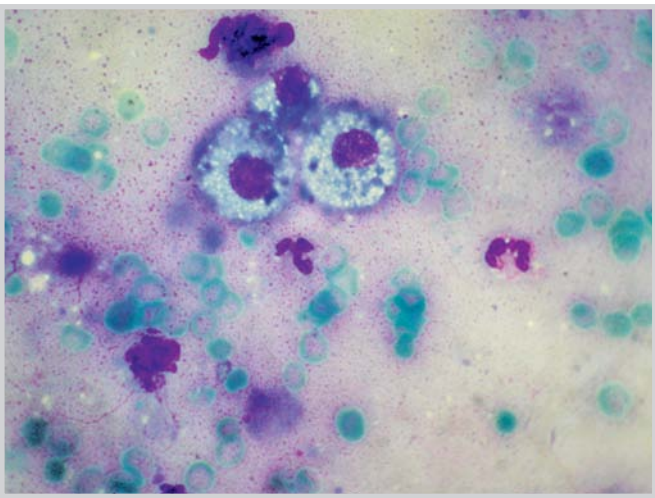

Fig. 27.56: Degenerated ductular cells, foam cells and polymorphs in nipple discharge smear (MGG × HP)

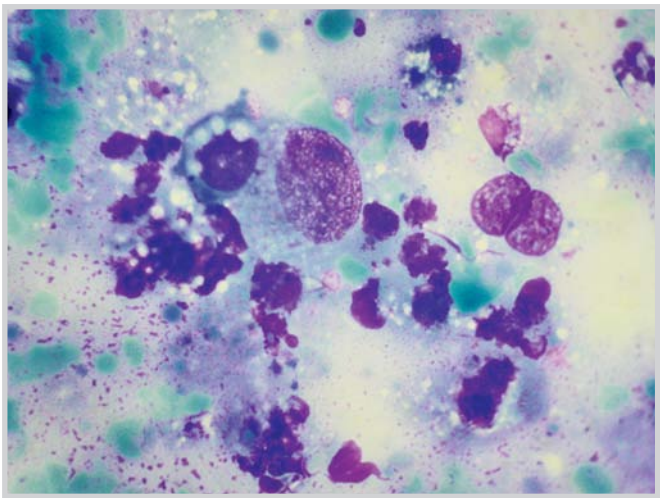

Fig. 27.57: Nipple discharge showing large cells with enlarge nuclei having multiple nucleoli in an infiltrating duct carcinoma (MGG × OI)

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CHAPTER 28

Lymph Node

Chapter Contents

INTRODUCTION

Fine needle aspiration cytology (FNAC) of the lymph node is now a widely applied technique for rapid and accurate diagnosis of various lesions. Peripheral lymph nodes are easily approachable and safe to aspirate. The common indications of FNAC of the lymph node are:

- Diagnosis and confirmation of clinically suspicious reactive lymphoid hyperplasia (RLH)
- Diagnosis of infective lymphadenopathy
- Diagnosis of metastatic malignancies
- Diagnosis, classification and staging of lymphomas
- Confirmation of the transformation of low to high grade of lymphoma in course of disease process.

Fine needle aspiration cytology is widely accepted to both the children and adult. It is a more or less painless procedure and does not need any anesthesia. The FNAC diagnosis is accurate and reliable. Presently, various ancillary investigations are possible from the FNAC material of the lymph node and these include microbiological culture, cell block for immunocytochemistry, flow cytometry, molecular genetics, etc.[1] Moreover, if necessary, lymph node can be excised for histopathological examination after FNAC report (**Box 28.1**).

There is no absolute contraindication of FNAC of lymph node. Local hematoma can be easily prevented by applying pressure on the aspiration site.

There are a few limitations of FNAC (**Box 28.2**). FNAC may not yield adequate or optimal material due to small size, fibrosis of the node, large areas of necrosis and in deep-seated lymph node. The other major limitation of FNAC of lymph node is the loss of architectural pattern. Lymph nodal architecture is helpful in diagnosis of certain lesions such as Castleman's disease, diffuse large B-cell lymphoma arising from the follicular lymphoma (FL), vascular transformation of lymph nodal sinuses, progressively transformed germinal center (GC), etc. Moreover, in a large research institute tissue section of the lymph node may be required to do correlative study and future research.

BOX 28.1 **Advantages of lymph node fine needle aspiration cytology (FNAC)**

- Rapid
- Economical
- Flow cytometric immunophenotyping
- Microbial and fungal culture
- Molecular genetics study
- Safe procedure

BOX 28.2 **Limitations of lymph node fine needle aspiration cytology (FNAC)**

- Sub optimal material:
  - Due to small size
  - Fibrosis of the node
  - Large areas of necrosis
- Loss of architectural detail:
  - Castleman's disease
  - Diffuse large B-cell lymphoma arising from the follicular lymphoma
  - Vascular transformation of lymph nodal sinuses
  - Progressively transformed germinal center (GC), etc.
- Tissue material may be needed for further research

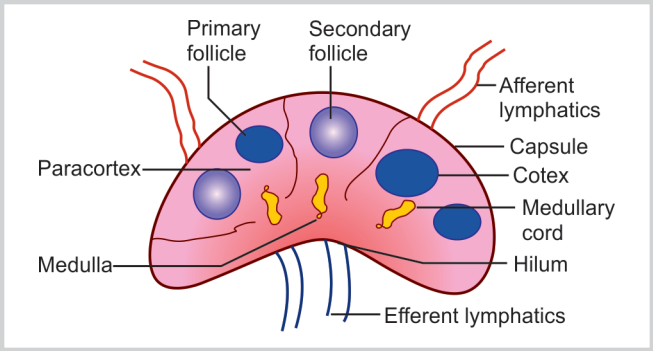


Fig. 28.1: Schematic diagram showing anatomy of the lymph node

NORMAL ANATOMY AND HISTOLOGY OF THE LYMPH NODE

Lymph node is a small bean-shaped encapsulated structure situated in the neck, axilla, inguinal region, body cavity and along the blood vessels. The other part of the lymph node is covered by capsule. The fibrous trabeculae of the capsule divide the lymph node into several small incomplete compartments. The afferent lymph vessels enter into the lymph node through the convex side of the lymph node and efferent lymph vessels emerge through the concave side where the hilum of the lymph node is situated.

Anatomically the lymph node is divided into three regions: (1) cortex, (2) paracortex and (3) medulla (**Fig. 28.1**).

Cortex: This is the subcapsular outer convex region of the lymph node. There are multiple lymphoid nodules (follicles) in the cortex. Two types of lymphoid nodules are seen: primary and secondary lymphoid nodules. The secondary lymphoid nodule contains pale GC. The lymphoid nodules contain B lymphocytes.

Paracortex: Paracortex is situated in between the cortex and medulla. T lymphocytes remain in the paracortex. Many high endothelial venules are located in this region.

Medulla: There are large tortuous lymphatic sinuses in the medulla. These sinuses are surrounded by cords of lymphoid cell known as medullary cords. The lymphocytes from the cortex often migrate from the cortex and enter the sinuses in medulla. From the medulla they enter the efferent lymphatic vessels and ultimately leave the lymph node.

Lymphoid cells: The ontogenic differentiation of B lymphoid cells has been described in detail in chapter 6, part 1 of this book (*see* Fig. 6.9).

B lymphocyte: B lymphocytes originally develop from pluripotent stem cells of bone marrow and pass through the several stages such as pro-B cell, pre-B cell, immature B cell and the mature B cells. The mature B cells migrate to the primary lymphoid follicle or nodule. During antigenic stimulation these mature B cells transform into centroblasts and form the GC into the secondary lymphoid follicles.

Morphologically, B cell undergoes a series of changes within the GC. The GC is a highly dynamic area and recently multiphoton laser scanning microscopy (MPLSM) is used to visualize the events in the GC.[2] B cells constantly move in the GC and dynamically interact with antigen and also T lymphocytes. The resting B cells undergo transformation to centroblasts to produce clones of plasma cells and memory B cells. The B cells transform from centroblasts, centrocytes and immunoblasts to plasma cells. The various populations of cells in the GC are:

Follicular Center Cells (Fig. 28.2)

- Small noncleaved cell (lymphoblast):
  - Small lymphocytes
  - Regular nuclear margin
  - Fine chromatin
  - Inconspicuous nucleoli
- Large noncleaved cell (centroblast):
  - Twice the size of the small lymphocytes
  - Fine nuclear chromatin
  - Multiple nucleoli
- Large cleaved cell (centrocytes):
  - Large cells with cleaved nuclei
  - Coarse chromatin
  - Small nucleoli
- Small cleaved cell (centrocytes):
  - Small cells
  - Larger than the mature lymphocytes
  - Cleaved nuclear margin
  - Coarse chromatin

Immunoblasts

Immunoblasts are developed in the GC and then migrate to the medullary cords. Here the cells complete their differentiation into plasma cells to produce antibody. Morphologic characteristic of the cells are:

- Large cells
- Three times larger than the mature lymphocytes
- Abundant deep basophilic cytoplasm
- Nuclei are round, regular
- Marginally placed prominent nucleoli

Plasma Cells

These are antibody producing cells. The morphological characteristics of the cells are:

- Small cells with eccentric nuclei
- Peripheral clumped chromatin giving rise to cart-wheel appearance
- Clear hallo around the nucleus (perinuclear hauff)

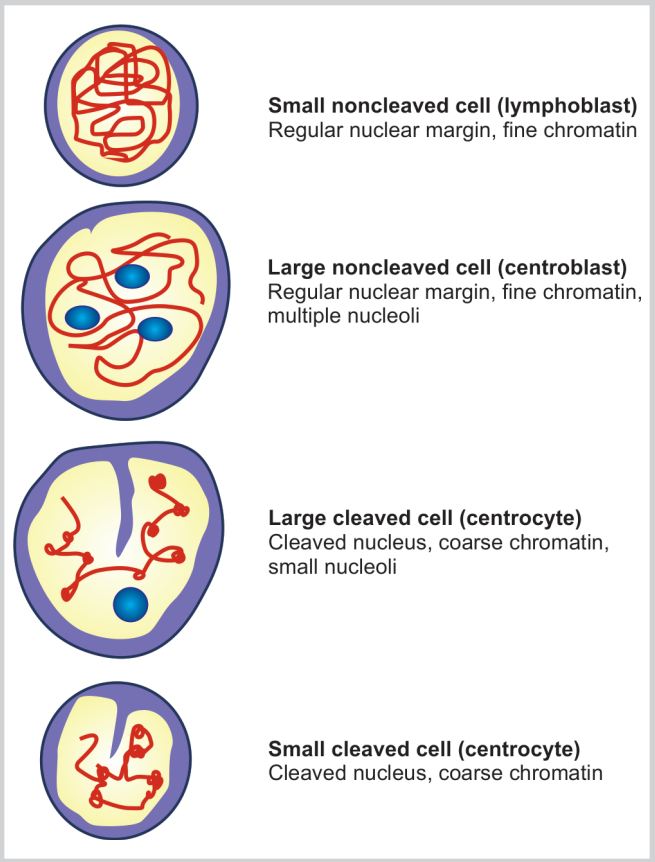


Fig. 28.2: Schematic diagram of different follicular center cells

Histiocytes

In addition, the lymph node also shows many histiocytes. The morphological characteristics of histiocytes are:

- Cells with abundant cytoplasm
- Centrally placed regular nuclei
- Reticular chromatin
- Variable amount of phagocytosed materials such as nuclear debris or pigments

Large histiocytes with many nuclear fragments are known as tingible body macrophages, are also seen in lymph node.

T Lymphocytes

T lymphocytes are small round to oval cell with scanty light blue cytoplasm having round nuclei with condensed chromatin. Morphologically, they are indistinguishable from B lymphocytes.

APPROACH OF LYMPH NODE FINE NEEDLE ASPIRATION CYTOLOGY

Clinical History

Before doing FNAC, proper clinical history is mandatory in each case. It is preferable to have clinical background of the patient and correlate the FNAC findings in the final interpretation. Most of the metastatic carcinoma occurs in elderly patient, whereas, lymphomas may occur in any age group. Duration of the enlarged lymph node is also important. Lymph nodal enlargement for long duration is unlikely to be a neoplastic process. Majority of the cases of lymphomas and metastatic carcinoma are of recent origin and rapidly progressive. Malaise and significant loss of body weight suggest the possibility of a neoplastic process. Evening rise of temperature and loss of weight are suggestive of tuberculosis. Any history of malignancy is helpful for the proper interpretation of FNAC.

Physical Findings

Site of lymph nodal enlargement is too some extent important. Most of the supraclavicular lymph nodes show metastatic carcinoma. In contrast, posterior cervical lymph nodes are frequently reactive in nature. FNAC, of inguinal lymph nodes is usually reactive in nature. Any lymph node more than 2 cm diameter is pathognomonic. Consistency of the lymph node is also very important. Indian rubber-like consistency is noted in Hodgkin's lymphoma (HL). Hard and fixed lymph node is suspicious for metastatic malignancy. Soft and tender lymph node is more commonly seen in inflammatory reaction.

Aspiration

Fine needle aspiration cytology of the lymph node can be done by the physician, surgeon and the pathologists. However, it is always preferable that cytologists should do FNAC procedure because at the time of FNAC, the pathologist may get a brief idea of the possibility of the diagnosis and on-site assessment of the aspiration smear may help to collect more samples for ancillary technique. FNAC of the peripheral lymph nodes does not deserve any local anesthesia. The overlying skin of the lymph node should be properly cleaned. The type of aspirated material may provide a lot of information. Necrotic material usually indicates an inflammatory disease. Thick-white caseous material strongly favors the possibility of tuberculosis. Turbid fluid is often aspirated in metastatic squamous cell carcinoma. The aspirated material should be transferred to buffered formalin for cell block to do immunocytochemistry. In case of suspicion of lymphoma, the pathologist should always take another pass to put the aspirated material in citrate buffer solution for flow cytometric immunophenotyping.[1] Similarly, the necrotic material should routinely be sent for bacterial or fungal culture.[3,4] In all cases, both the air-dried smear and alcohol-fixed wet smear should be made for May-Grunwald-Giemsa (MGG) and Papanicolaou's stain, respectively. MGG stained smear gives better idea in hemtolymphoid tumors.

NORMAL COMPONENT OF A LYMPH NODE (Box 28.3)

The detailed description of these cells has been discussed in earlier paragraphs in this chapter.

Small bluish cytoplasmic fragments of the lymphoid cell are seen on the background. These bluish fragments are known as lymphoglandular bodies (LG) (**Fig. 28.3**).

BOX 28.3 Normal components of a lymph node

- Mature lymphocytes
- Centrocytes
- Centroblasts
- Immunoblasts
- Plasma cells
- Polymorphs
- Eosinophils
- Mast cells
- Tingible body macrophages.

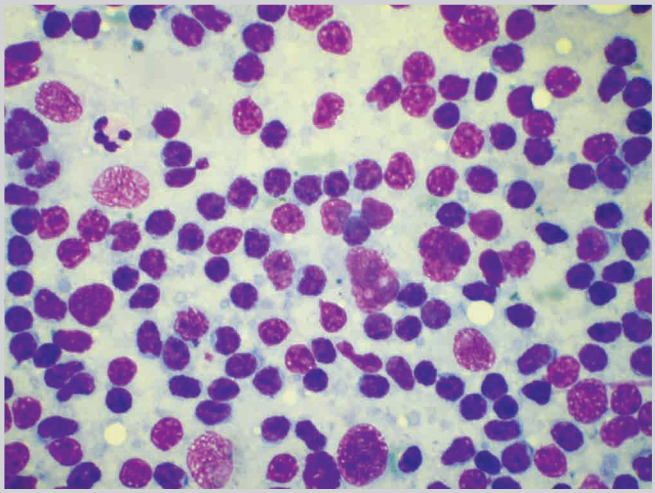


Fig. 28.3: Fine needle aspiration cytology (FNAC) smear of reactive lymphoid hyperplasia. Note the bluish acellular fragments known as lymphoglandular bodies [May-Grünwald-Giemsa (MGG) stain × oil immersion (OI)]

DIAGNOSTIC ACCURACY

The diagnostic accuracy of FNAC of lymph node largely depends on:
(1) the adequacy of material, (2) experience of the cytopathologist, (3) type of the malignancy and (4) use of ancillary techniques such as flow cytometry, immunocytochemistry and study of molecular genetics. FNAC material is limited or suboptimal in case of predominantly necrotic or fibrotic lymph node and accurate diagnosis may be difficult in such conditions. Similarly, in case of subcapsular deposition of the malignant cells, FNAC may give false negative report.

Diagnostic accuracy of FNAC is near about 90–95% in metastatic malignancy in the lymph node.[5,6] False positive report of metastatic malignancy is also very rare.[5] However, the cytopathologist should be careful regarding the false positive report due to inclusion of skin adnexal glands in axillary lymph node of breast carcinoma cases. Similarly, benign salivary epithelial cells may also be included in the lymph node aspirate of neck gland.

The diagnostic accuracy of lymphomas is variable and overall it is near about 80%, if the diagnosis is based only on cytomorphology. FNAC can provide a reliable and conclusive diagnosis in high grade non-Hodgkin's lymphoma (NHL) and HL. However, the diagnosis of low grade NHL is often difficult. There may be considerable difficulty in cytological diagnosis of low grade NHL particularly small lymphocytic lymphoma (SLL). Similarly, lymphocytic predominant HL is difficult to diagnose on FNAC. In contrast, high grade NHL such as Burkitt's lymphoma (BL) and lymphoblastic lymphomas are relatively easy to diagnosis. The use of ancillary techniques particularly flow cytometry and immunocytochemistry increases the diagnostic accuracy rate. In fact, accurate flow cytometric immunophenotyping greatly helps in the subtyping of NHL.[7]

Fine needle aspiration cytology is highly accurate (more than 90%) in diagnosis of metastatic malignant lesions of the lymph node. The majority of lymphomas can be diagnosed on FNAC material. However, the accuracy of the classification of lymphoma is variable in aspiration material. The overall accuracy rate of lymphoma is near about 95–99% and typing accuracy rate is 96%.[8-12] FNAC can provide a reliable and conclusive diagnosis in high grade NHL and HL. However, the diagnosis of low-grade NHL is often difficult. Different ancillary techniques are very helpful to help in this aspect.

BENIGN LESIONS IN THE LYMPH NODE

Reactive Lymphoid Hyperplasia

Reactive lymphoid hyperplasia (**Figs 28.4** to **28.8**) is frequently encountered in FNAC of lymph node. Patients may be in any age group but the children more commonly show enlarged reactive lymph node than the elderly population. The neck, axilla and inguinal regions are more frequently involved. There are various causes of RLH, such as viral infection, bacterial infection, drainage area of cancer site and any sort of antigenic stimulation (**Box 28.4**).

Cytology

FNAC usually yields high cellularity in reactive lymphoid hyperplasia (RLH). The smears show abundant discrete polymorphic population of lymphoid cells consisting of large number of mature lymphocytes, follicular center cells (centrocytes and centroblasts), immunoblasts, plasma cell, polymorphs, eosinophils and tingible body macrophages. The tingible body macrophages are large histiocytes with phagocytosed nuclear debris. They represent the cell turnover in the smear. Background of the smear shows LG. The presence of LG body indicates only the presence of lymphoid tissue. It has no other significance. RLH shows aggregate of lymphoid cells and histiocytes known as lymphohistiocytic tangles. The lymphohistiocytic aggregates are the hall mark of RLH. Frequent mitotic activities are also seen as the lymphoid cells always get mitogenic stimulation and undergo proliferation. It should always be remembered that abundant cellularity and mitosis are not the criteria of malignancy in the FNAC of lymph node.

The cytopathologist often faces difficulty to differentiate RLH and low grade NHL. Small lymhocytic lymphoma or mantle cell lymphoma (MCL) shows small monomorphic population

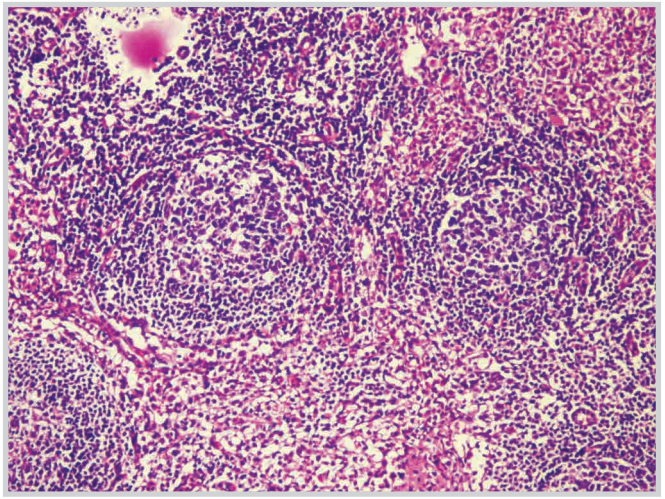


Fig. 28.4A: Histopathology section of reactive lymphoid hyperplasia [hematoxylin and eosin (H & E) stain × Medium power (MP)]

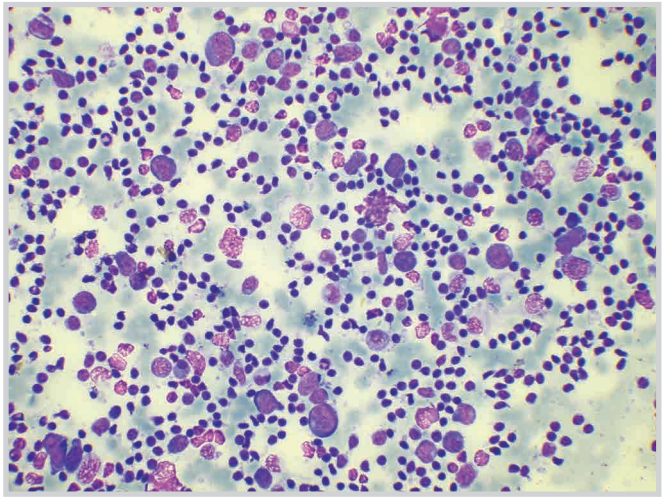


Fig. 28.4B: Polymorphic population of cells in reactive lymphoid hyperplasia [(MGG × High power(HP)]

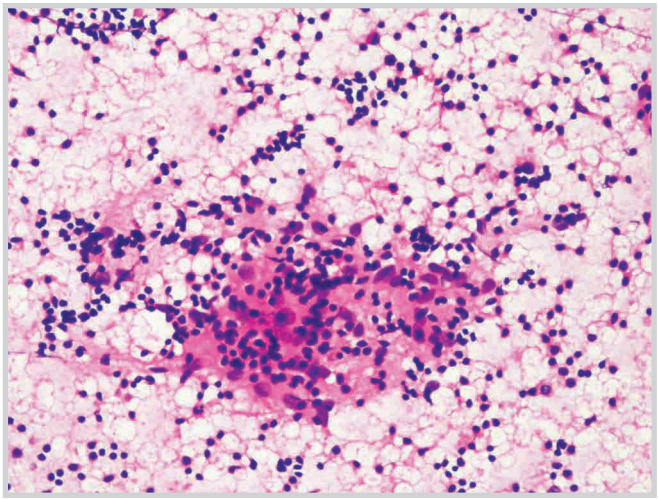


Fig. 28.5: Lympho-histiocytic aggregate in reactive lymphoid hyperplasia (MGG ×MP)

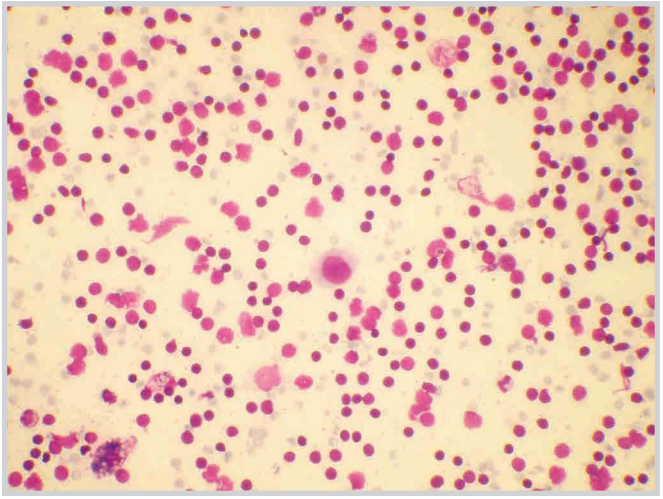


Fig. 28.6: Large atypical lymphoid cells in a case of viral infection (MGG × MP)

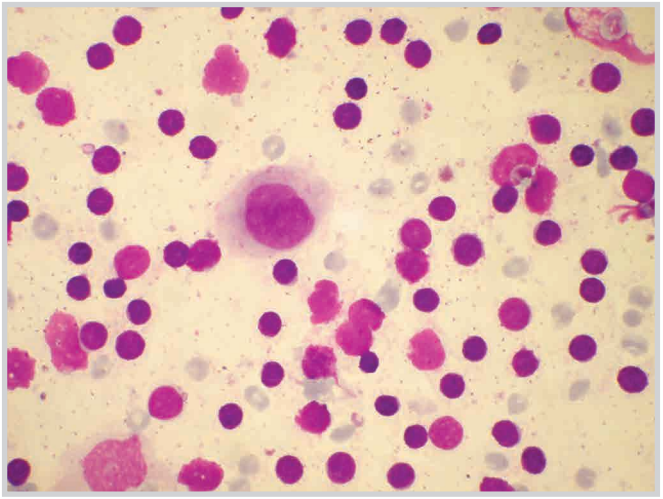


Fig. 28.7: Higher magnification of the previous smear. Note the enlarged lymphoid cell with moderate amount of cytoplasm (MGG × HP)

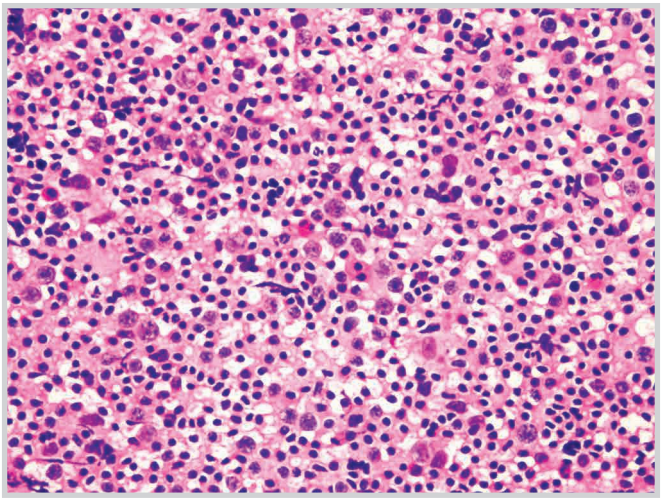


Fig. 28.8: Abundant immunoblasts in a case of reactive lymphoid hyperplasia (H & E × HP)

BOX 28.4 Reactive lymphoid hyperplasia (RLH)

- Polymorphic population
- Lymphocytes
- Centrocytes
- Centroblasts
- Immunoblasts
- Plasma cells
- Occasional neutrophils, eosinophils, mast cells
- Tingible body macrophages
- Lymphohistiocytic tangles
- Lymphoglandular bodies

of lymphoid cells and at times difficult to differentiate from RLH. Relatively polymorphic population of cells, tingible body macrophages, and lymphohistiocytic tangles favor RLH. Along with the cytological features, clinical details of the patients are also helpful in distinguishing reactive changes versus NHL. Occasional cases may need demonstration of light chain restriction or aberrant T cell markers expression to confirm the diagnosis of NHL.

Abundant population of reactive lymphoid cells and the presence of occasional Reed-Sternberg (RS) cell in HL are often confused with RLH particularly in early evolving HL and lymphocyte predominant cases.

Viral infections particularly infectious mononucleosis (IM) infection may show large atypical cells. These cells are large with abundant basophilic cytoplasm and eccentric nuclei. The atypical cells are also associated with transformed lymphocytes. These cases may be misdiagnosed as malignancy. However, a good clinical history is helpful to avoid any false positive diagnosis.

At times, FNAC smears of RLH may show abundant number of immunoblasts and this may misled the cytologist to the wrong diagnosis of lymphoma or nasopharyngeal carcinoma.

Rarely, FNAC of small quiescent lymph nodes may show small monomorphic population of lymphoid cells mimicking small lymphocytic lymphoma (SLL). One should be careful about not to misinterpret such cases.

Differential Diagnosis

- Non-Hodgkin's lymphoma
- Hodgkin's lymphoma
- Benign lymphadenopathy: Castleman's disease, dermatopathic lymphadenitis and HIV-associated lymphadenopathies.

Acute Lymphadenitis

Clinically, the patients of acute lymphadenitis present with tender enlarged lymph node. The nodes are inflamed, red and hot. The patients may have fever. Acute lymphadenitis is usually noted in the drainage area of acute abscess and predominantly is caused by bacterial infection.

Cytology

FNAC smears show abundance of neutrophils and other inflammatory cells along with background necrosis. Later on aspirate of the node yield purulent material.

BOX 28.5 Granulomatous lymphadenitis

- Multiple epithelioid cell granulomas
- Multinucleated Langhans type giant cells
- Necrosis
- Reactive lymphoid cells

Granulomatous Lymphadenitis[13,14]

Granulomatous lymphadenitis occurs in a variety of infective conditions, foreign body reactions and sarcoidosis (**Box 28.5**). The detailed causes of granulomatous inflammation have been discused in chapter 3. In the Indian subcontinent tuberculosis is the most common cause of granulomatous lymphadenitis.

Tuberculosis

Tuberculosis is the most commonly caused by *Mycobacterium tuberculosis infection*. The other two mycobacteria causing tuberculosis are *Mycobacterium bovis* and *Mycobacterium africanum*. Due to pasteurization of milk, *Mycobacterium bovis* infection is almost rare in human. Nontuberculous mycobacteria (NTM) infection is now on the rise in both developed and developing countries. *Mycobacterium scrofulaceum*, *Mycobacterium avium-intracellulare* complex and *Mycobacterium kansaii* are three common NTM. Out of these, *Mycobacterium avium-intracellulare* is the most common causative agent of nontuberculous adenitis.

Tuberculosis usually indicates pulmonary tuberculosis. The isolated involvement of other body sites by tuberculosis is known as extrapulmonary tuberculosis. Tuberculous lymphadenitis is the most common site of extrapulmonary tuberculosis and represents 35% all extrapulmonary tuberculosis.[15] The tuberculous lymphadenitis may occur in any age group. However, this commonly affects the patients between 20–40 years. The patients typically present with painless enlarged lymph node. Systemic symptoms such as fever, malaise and loss of body weight may or may not be present.

The tuberculous infection of the lymph node most likely occurs due to spread of the bacilli through lymphatic from the lung parenchyma.[16] However, the other routes of spread to lymph nodes, such as the tonsils and adenoids, have been hypothesized.[17]

Cytology (Figs 28.9 to 28.12)

FNAC smears of tuberculous lymphadenitis show multiple epithelioid cell granulomas, multinucleated Langhans type of giant cell and reactive lymphoid cells in a necrotic background. The epithelioid cell granuloma consists of well circumscribed aggregation of epithelioid cells and lymphocytes. The individual epithelioid cell show moderate amount of pale distinct cytoplasm along with spindle-shaped nuclei with blunt ends. The multinucleated Langhan's giant cells contain abundant cytoplasm with multiple nuclei that are arranged in horseshoe shaped manner. The total number of nuclei is 15–30. The shape of nuclei may be round to spindle-shaped. The reactive lymphoid population shows follicular center cells, mature lymphocytes, plasma cells, eosinophils and immunoblasts. FNAC of

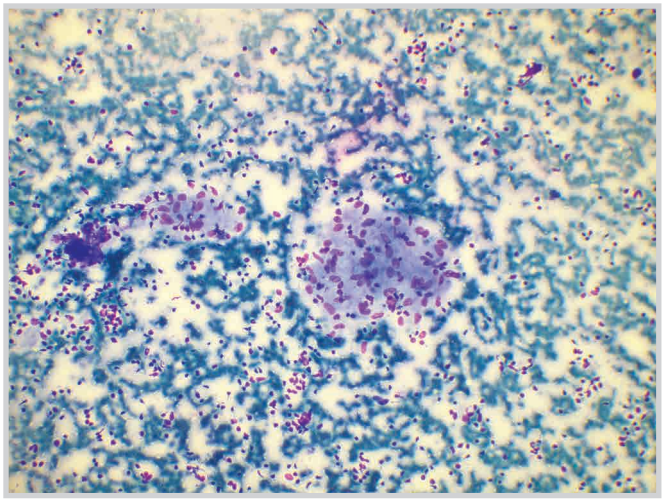


Fig. 28.9: Multiple epithelioid cell granulomas in a background of reactive lymphoid cells in tuberculous lymph node (MGG × MP)

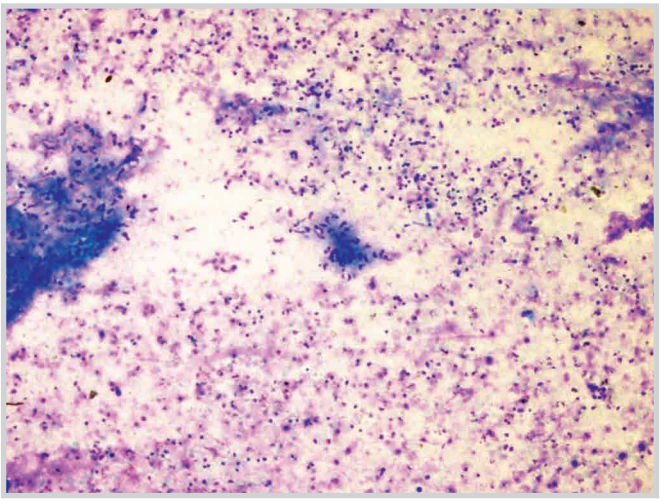


Fig. 28.11A: Epithelioid cell granuloma in the background of necrosis in tuberculous lymph node [(MGG × Low power(LP)]

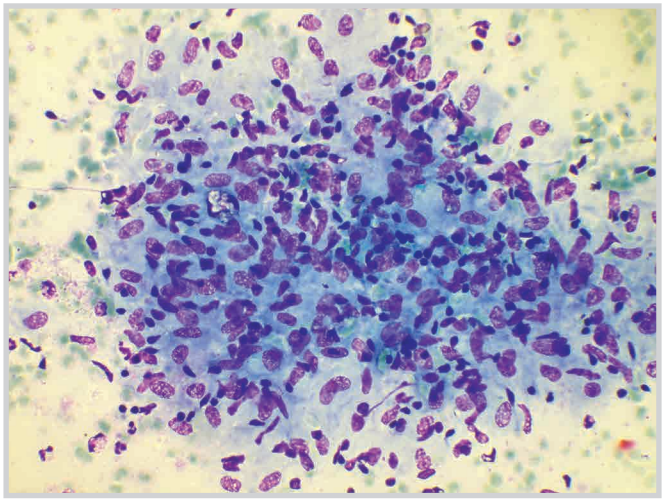


Fig. 28.10: Higher magnification showing the epithelioid cell granuloma in tuberculous lymph node. Note the close admixture of lymphocytes with the epithelioid cells in this granuloma (MGG × OI)

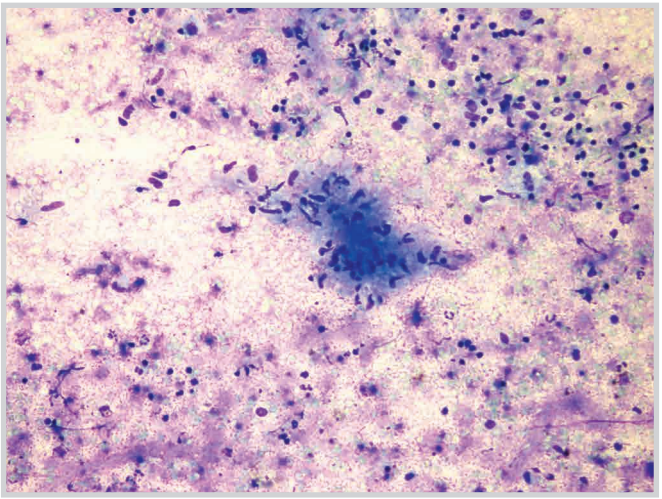


Fig. 28.11B: Higher magnification showing epithelioid cell granuloma, polymorphs and histiocytes (MGG × HP)

tuberculous lymphadenitis may present as following patterns of smears:

- Predominantly reactive lymphoid cells and occasional epithelioid cell granulomas: In this type, the abundant reactive lymphoid cells may mislead the pathologists and diagnosis may be missed unless a thorough search is made.
- Epithelioid cell granulomas and multinucleated giant cells in a necrotic background: This type of smear is readily identifiable for tuberculous infection.
- Only necrosis: The smear shows only necrotic cells. There may not be any evidence of tuberculosis other than the demonstration of acid-fast *bacilli* (AFB) in Zeihl-Neelsen (Z-N) stain.
- Only reactive lymphoid cells: This is the most uncommon type of smear pattern and may be noted in HIV infected patients due to lack of any immunological response.

Hodgkin's lymphoma often shows multiple epithelioid cell granulomas. Demonstration of typical RS cell is helpful in confirmation of HL. The epithelioid cell granulomas in HLs are

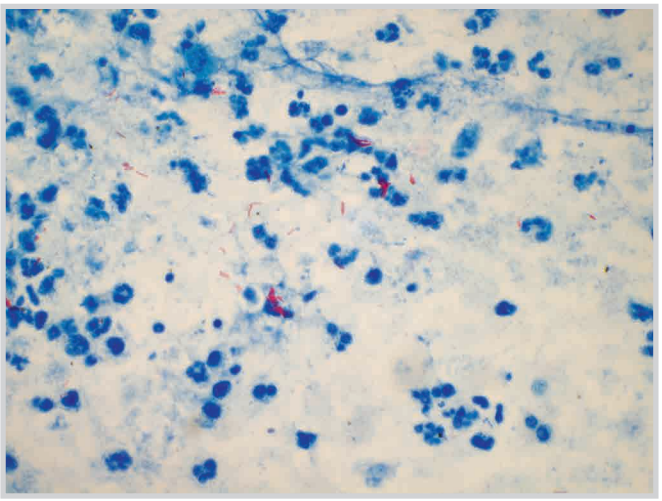


Fig. 28.12: Many acid fast bacilli in the necrotic background. (Ziehl Neelsen stain × oil immersion)

BOX 28.6 **Epithelioid cell granulomas noted in malignancies**

- Hodgkin lymphomas(HL)
- T cell rich-B cell lymphomas
- Seminoma
- Metastatic squamous cell carcinomas

BOX 28.7 **Sarcoidosis**

- Multiple epithelioid cell granulomas
- Multinucleated giant cells
- No necrosis
- Reactive lymphoid cells
- Negative for acid fast bacilli (AFB) or fungi

BOX 28.8 **Leprosy lymphadenitis**

- Foamy multivacuolated cells
- Multinucleated giant cells
- Epithelioid cell granuloma
- Modified Z-N stain: bundles of intracytoplasmic lepra bacilli

usually ill-formed. Certain lymphomas such as T cell-rich B cell lymphomas show epithelioid cell granulomas. Background immature lymphoid cells are helpful diagnostic clue in these cases. Epithelioid cell granulomas are also seen in seminoma and metastatic squamous cell carcinomas (**Box 28.6**).

Demonstration of AFB: In each suspected cases of tuberculous lymphadenitis, Z-N stain should always be done to demonstrate mycobacterium. The sensitivity of Z-N stain for the detection of mycobacteria is 33–59% and specificity is 100%.[13,14] Usually, smears with necrosis and epithelioid cell granulomas have the highest chance of AFB positivity. The overall rate of isolation of mycobacteria on culture is 35–49%.[18] The combined use of Z-N stain of the direct smear and mycobacterial culture of aspirated material increases the sensitivity of detection of mycobacteria.[13] Nowadays many newer culture techniques of mycobacteria are available such as the BACTEC 460 radiometric system, BACTEC MGIT 960 system, MB/BacT system, ESP II culture system and microcolony detection on solid media. These techniques improve mycobacterial isolation rate and substantially reduce detection time as compared with culture in Löwenstein-Jensen (L-J) medium.[19, 20]

Polymerase chain reaction (PCR) on the aspirated material increases the sensitivity of the detection of mycobacteria. The sensitivity and specificity of PCR in FNAC material of tuberculous lymphadenitis are 84% and 75%, respectively. It has been noted that the combined use of FNAC smear and PCR increases the sensitivity and specificity to 84% and 100%, respectively.[21]

Differential Diagnosis

- Hodgkin's lymphoma
- Non-Hodgkin's lymphoma
- Fibroblasts

Sarcoidosis

Sarcoidosis is a systemic disease of unknown etiology. It occurs in all age group but most commonly is seen in third to fourth decade. The disease invariably involves lung and thoracic group of lymph nodes.[22] Peripheral lymph nodes may also be involved particularly in head and neck region.[23] The diagnosis of the sarcoidosis depends on the clinical, radiological and histopathological features. Noncaseating epithelioid cell granuloma in the absence of any organism is suggestive of sarcoidosis.

Cytology

FNAC smear of sarcoidosis shows multiple noncaseating epithelioid cell granulomas, multinucleated giant cells and lymphoid cells (**Box 28.7**). The giant cells may rarely contain calcium oxalate crystals and asteroid bodies.[24] The epithelioid cell granuloma of sarcoidosis is indistinguishable from the granulomas of other diseases. However, the cytology smear in sarcoidosis does not show any organisms or necrosis.

Differential Diagnosis

Other granulomatous disease

Lepromatous Lymphadenitis

Fine needle aspiration cytology is rarely done in lepromatous lymphadenitis cases. The cytology smears show many foamy histiocytes arranged discretely or in loose clusters (**Box 28.8**). The individual cells have abundant multivacuolated soap bubble type of cytoplasm. The nuclei are centrally placed with fine chromatin and prominent nucleoli. Intracytoplasmic unstained areas may also be noted. In addition, the cytology smears show multinucleated giant cells and epithelioid cell granulomas. The number or abundance of epithelioid cell granulomas depends on the type of the disease. Tuberculoid leprosy will have more number of epithelioid cell granulomas than lepromatous leprosy.[25] Modified Z-N staining of the smear shows bundles of lepra bacilli within the histiocytes.

Leishmania Lymphadenitis

Leishmaniasis is caused by protozoa of the genus *Leishmania*. Visceral leishmanias is caused by *Leishmania donovani* infection. The sandfly inoculates promastigote form of *Leishmania* into the skin. These promastigote forms of *Leishmania* are phagocytosed by the macrophages. The promastigotes are transformed into amastigotes within the histiocytes. The amastigotes come out from the histiocytes by rupturing the cells. These amastigotes multiply in the reticuloendothelial cells of the body. Lymph nodal involvement of *Leishmania* is rare.

Cytology[26]

Cytology smear of leishmanial lymphadenitis shows mixed population of cells consisting of lymphocytes, plasma cells, histiocytes. Occasional multinucleated giant cells and epithelioid cell granulomas may be noted.[27] Within the histiocytes, multiple amastigotes are noted. These are 1 µm–3 µm diameter oval structures with a fine membrane and a relatively large nucleus. Many parasites may remain free in the background of air-dried smear.

Filarial Lymphadenitis

Wuchereria bancrofti may cause lymphadenopathy in tropical countries (**Figs 28.13** and **28.14**). The microfilaria parasites may occasionally be noted in the FNAC of the lymph node. The smears show the extracellular long-sheathed parasite with a pointed tail that is free of nuclei. In addition, the smear may show eosinophils, reactive lymphoid cells and epithelioid cell granulomas.[27]

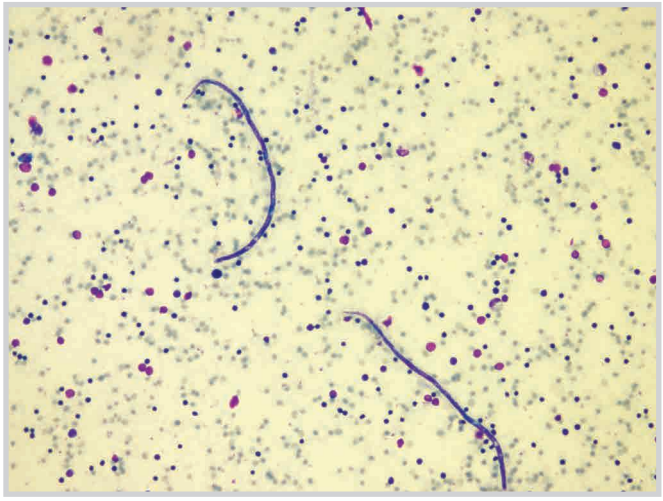


Fig. 28.13: Microfilaria in a background of reactive lymphoid cells (MGG × LP)

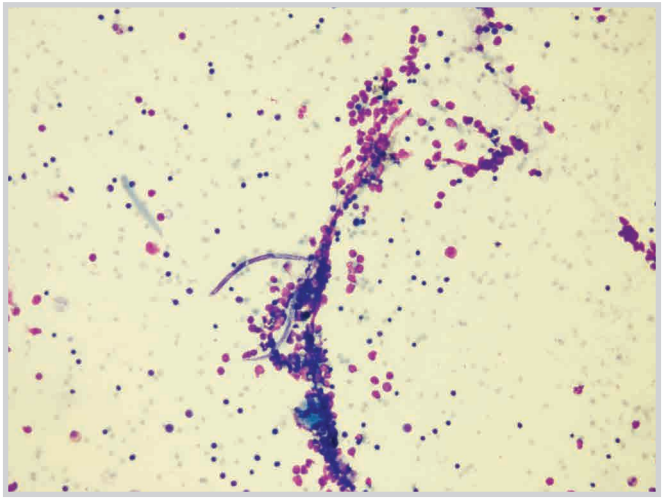


Fig. 28.14: Microfilaria in higher magnification (MGG × MP)

Toxoplasma Lymphadenitis

Toxoplasmosis is a zoonotic infection. Feline such as cat is the definitive host and man is intermediate host. In human, it is caused by an intracellular parasite, *Toxoplasma gondii*. FNAC of toxoplasma lymphadenitis shows necrosis, reactive lymphoid cells and large histiocytes with finely granular cytoplasm. Within the necrotic exudate numerous small, elongated, and crescent-shaped organism may be seen. This is best visualized by MGG stain. *Toxoplasma gondii* may also be present within the histiocytes.

Rosai-Dorfman Disease[29-31]

Rosai-Dorfman disease is also known as sinus histiocytosis with massive lymphadenopathy (SHML) (**Box 28.9**). SHML is commonly seen in the first and second decade of life. However, it may affect the patient in age group. The patient of SHML presents with painless massive bilateral cervical lymphadenopathy, fever, joint pain, loss of weight, leukocytosis, elevated erythrocyte sedimentation rate (ESR) and polyclonal hypergammagloubilinemia. Clinically, the symptoms and signs of the patient may often mimic lymphoma. In 25% of cases, there may be extranodal involvement of SHML and include salivary glands, ocular adnexa, bone and skin regions. Extranodal involvement of SHML is usually associated with massive enlargement of the lymph node.[32] SHML is a self-limiting condition and regress spontaneously.

Cytology (Fig. 28.15)

Cytology smears of SHML exhibit polymorphic population of cells consisting of many dissociated large histiocytes, plasma cells and

BOX 28.9 Rosai-Dorfman disease

- Reactive lymphoid cells
- Discrete large histiocytes with abundant pale cytoplasm
- Central round, pleomorphic nuclei
- Phagocytosed lymphocytes and plasma cells (emperipolesis)

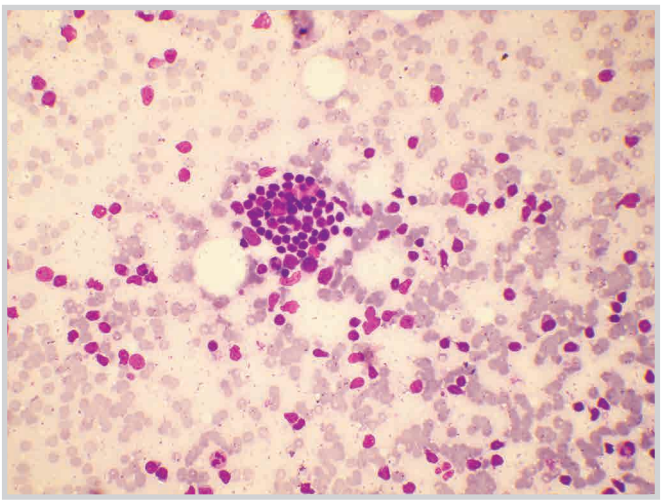


Fig. 28.15: Phagocytosed lymphocytes known as emperipolesis in Rosai-Dorfman disease (MGG ×X MP)

reactive lymphoid cells. These large histiocytes have abundant pale cytoplasm and centrally placed nuclei with prominent nucleoli. Nuclear enlargement and pleomorphism is also noted. The histiocyte characteristically contains phagocytosed lymphocytes and plasma cells. This phenomenon is known as emperipolesis.[29,30] Even polymorphs may be phagocytosed by this histiocyte. There remains a clear halo around the phagocytosed blood cells.

Due to the presence of abundant reactive lymphoid cells, SHML may simulate as reactive lymph nodal hyperplasia. However, the characteristics emperipolesis is a clue to diagnosis along with clinical features of massive lymph nodal enlargement.

Nuclear enlargement and mild pleomorphism of the histiocytes in SHML may simulate malignant histiocytosis particularly in the extranodal sites.[31] However, the nuclear atypia is more marked in case of malignant histiocytosis. The presence of abundant histiocytes in SHML may mimic Langerhans cell histiocytosis (LCH). The prominent nuclear grooves in the histiocytes, large number of eosinophils and absence of emperipolesis are important differentiating cytological features of LCH from SHML.

The hemophagocytosis syndrome may also present with lymph nodal enlargement and FNAC smear shows phagocytosis of red blood cells and platelets whereas, SHML shows phagocytosis of lymphocytes and plasma cells.

Immunocytochemistry

The histiocytes are positive for CD 68 and S-100 , CD11c, CD14, CD33 immunostaining and negative for CD 1a.[33]

Differential Diagnosis

- Reactive lymphoid hyperplasia
- Malignant histiocytosis
- Langerhans cell histiocytosis
- Hemophagocytosis syndrome
- Lymphoma

Kimura's Disease[34]

This is an inflammatory condition of the lymph node with unknown etiology. The patient presents with involvement of lymph nodes in the head and neck region, salivary gland enlargement and subcutaneous manifestation. This is an uncommon entity in Indian subcontinent.

Cytology

Histology section of Kimura's lymph node shows reactive follicular hyperplasia with well vascularized germinal centers containing polykaryocytes and eosinophilic abscess (**Box 28.10**). Cytology smear shows abundant eosinophils, reactive lymphoid cells, endothelial cells and occasional multinucleated giant cells known as Warthin- Finkeldey giant cells. These findings are nonspecific; however, the presence of excessive eosinophils in a clinical setting is suggestive of Kimura's disease.

Polymorphic population of cells along with background of abundant eosinophils may pose diagnostic difficulty with HL. However, classical RS cell is the diagnostic feature of HL and usually these cells are readily identifiable on cytology smear. The numerous eosinophils in Kimura's disease may simulate a parasitic infection and thorough search for parasites should be done.

Differential Diagnosis

- Parasitic infection
- Hodgkin's lymphoma

Kikuchi's Disease[35]

Kikuchi's disease is commonly noted in Japan and other Asian countries. However, it may also occur in USA and Europe. The patient is usually young woman and presents with fever and painless cervical lymphadenopathy. This is a self-limited disease.

Cytology

On histology, the section of the lymph node shows well defined paracortical necrotizing lesions. Characteristically the lesion is devoid of any neutrophil and plasma cells.

The cytology smear shows abundant karyorrhectic debris and histiocytes (**Box 28.11**). There is remarkable absence of polymorphs and plasma cells. The histiocytes show angulated crescent-shaped eccentric nuclei that are peripherally pushed.[35] In a proper clinical setting, the diagnosis of Kikuchi's lymphadenitis may be possible on cytology smear.

Marked necrosis in granulomatous inflammation may mimic Kikuchi's disease. However the absence of polymorphs and the crescent-shaped nuclei of the histiocytes are helpful diagnostic features of Kikuchi's disease. Epithelioid histiocytes in granulomatous reaction have plump-shaped nuclei with smooth outer margin.

Differential Diagnosis

- Necrotizing granulomatous lymphadenitis
- Reactive lymphoid hyperplasia with abundant tingible body macrophages

BOX 28.10 Kimura's disease

- Polymorphic population
- Lymphocytes, histiocytes
- Numerous eosinophils forming abscess

BOX 28.11 Kikuchi's lymphadenitis

- Necrotic debris
- Histiocytes with angulated crescent-shaped eccentric nuclei
- Almost complete absence of polymorphs and plasma cells

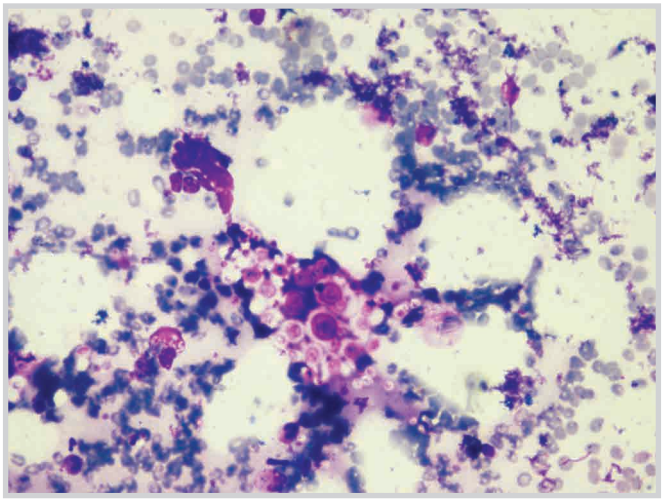


Fig. 28.16: Round encapsulated cryptococcus fungi in human immunodeficiency virus (HIV) lymphadenopathy case (MGG × OI)

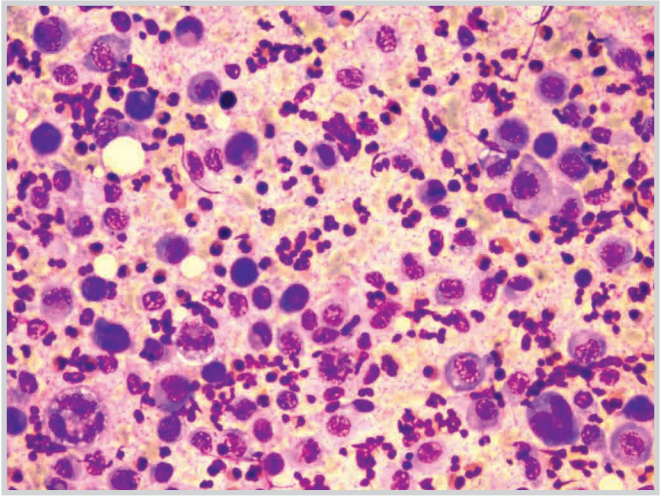


Fig. 28.17: Abundant mono and multinuclear histiocytes along with background eosinophils in Langerhans cell histiocytosis of lymph node (MGG × MP)

Human Immunodeficiency Virus Lymphadenopathy

Lymphadenopathy is one of the earliest manifestations of human immunodeficiency virus (HIV) infections. The predominant type of lymph nodal lesions in HIV are:[36]

- Reactive lymphoid hyperplasia
- Various opportunistic infections
- Kaposi sarcoma
- Non-Hodgkin's lymphoma
- Hodgkin's lymphoma

Out of all these type of lesions, the most common lesion is florid RLH.

Cytology[37-39] (Fig. 28.16)

There is persistent generalized lymphadenopathy (PGL) and the cytology smears show predominantly florid reactive lymphoid cells consisting of follicular center cells, immunoblasts and monocytoid B cells having relatively abundant, clear cytoplasm. It is not possible to differentiate HIV-induced PGL from a simple RLH on FNAC sample.

Later on, in the "burn out" phase, there are numerous plasma cells along with scanty tingible body macrophages, and follicular center cells. In all such cases, a thorough search is needed to find out any fungi. Z-N stain should always be done to exclude the possibility of tuberculosis even in the background of RLH.

Langerhans Cell Histiocytosis[40,41]

Langerhans cell histiocytosis is a systemic disorder that commonly involves bone, skin, lymph nodes, bone marrow, lungs, hypothalamic pituitary axis, spleen and liver. The disease is characterized by the clonal proliferation of Langerhans cell, a distinct type of immune "accessory" cells. The patients are usually children; however no age group is exempted from this lesion.

Cytology (Figs 28.17 and 28.18)

The cytology smears show many mononuclear and multinucleated histiocytes, reactive lymphoid cells and abundant eosinophils

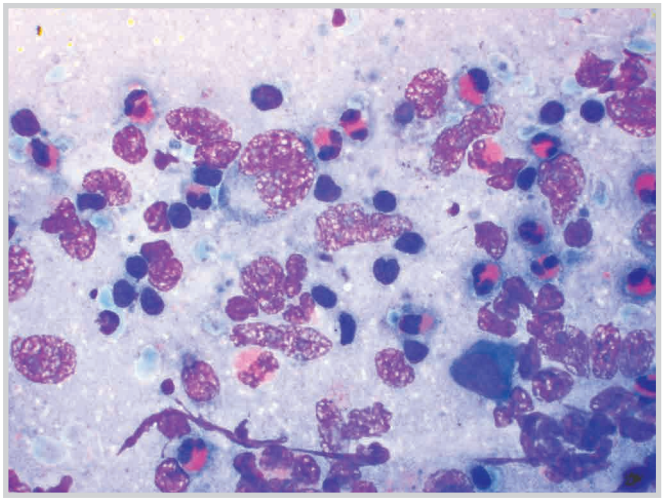


Fig. 28.18: Higher magnification showing deep nuclear convolution of the histiocytes in Langerhans cell histiocytosis of lymph node (MGG × OI)

BOX 28.12 Langerhans cell histiocytosis

- Abundant histiocytes
- Mono and multinuclear
- Oval to bean-shaped nuclei, with characteristic linear folds or grooves in the nuclear membrane
- Many eosinophils
- Immunocytochemistry: S-100 and CD1a positive. CD68 negative
- Electron microscopy: Birbeck granules present

(**Box 28.12**). The histiocytes contain abundant cytoplasm with centrally placed nuclei. The nuclei are characteristically bean-shaped with deep longitudinal nuclear grove. The cytoplasm of the histiocytes is usually free from any phagocytosed material. In addition, the smear shows abundant eosinophils. Large number of eosinophils may cause diagnostic difficulty with parasitic or

fungal infections. The presence of deep nuclear grooves in the histiocytes is characteristic of LCH.

Immunocytochemistry

Langerhans cells are positive for S 100 and CD1a.

Electron microscopy: Positive for Birbeck granules.

Differential Diagnosis

Parasitic or fungal infection

Dermatopathic Lymphadenitis

This is commonly seen in the drainage area of a dermatitis lesion such as including psoriatic erythroderma, exfoliative dermatitides and mycosis fungoides.

Cytology (Fig. 28.19)

The cytology smear shows a reactive lymphoid cell population consisting of follicular center cells, lymphocytes, and immunoblasts along with many histiocytes containing dark-brown melanin pigments. Many plasma cells are often seen in the background. The dermatopathic lymphadenitis should be distinguished from metastatic melanoma because of melanin containing cells in both the lesions. However, the nuclei of the melanin-containing cells are benign in look in case of dermatopathic lymphadenitis.

Differential Diagnosis

Metastatic melanoma

Castleman's Disease

This is also known as angiofollicular lymph node hyperplasia. This is a distinct form of lymph nodal hyperplasia, the etiology of which is unknown. Castleman's disease commonly occurs in adults, however rarely children may be affected. The patient presents with localized or generalized lymphadenopathy. Mediastinal lymph nodes are commonly enlarged. There are two histological types of Castleman's disease: (1) hyaline vascular type and (2) plasma cell type. Hyaline vascular type accounts for approximately 90% cases, whereas, the plasma cell variant is much less common and is associated with multifocal lymphadenopathy and systemic symptoms.

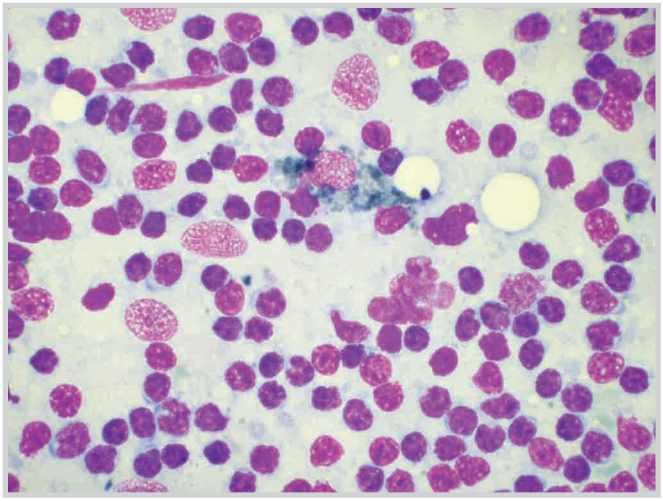


Fig. 28.19: Histiocyte containing melanin pigment in dermatopathic lymphadenitis (MGG × HP)

Cytology

Cytology smears of Castleman's lymph node show abundant discrete polymorphic population of lymphoid cells admixed with follicular dendritic cells and tingible body macrophages. Eosinophilic granular material and hyalinized capillaries are seen within the follicular center cells. Follicular dendritic cells are large with abundant pale cytoplasm having ill-defined outlines. The nuclei are variable sized and often convoluted giving a coffee bean appearance. Nuclear chromatin is fine with small chromocenter. Occasionally, the nuclei show coarse chromatin with an appearance of a wrinkled tissue paper. Most of the time the nuclei of such cells are monomorphic, however, occasionally large atypical nuclei may be seen. These cells may be mistaken for RS cells of Hodgkin disease.[42-44]

The recognition of Castleman's disease on FNAC smear is difficult because the characteristic hyaline vascular appearance can only be seen in histopathology section. However, in appropriate clinical setting, the certain cytomorphological features may be helpful in recognition of Castleman's disease.

Differential Diagnosis

- Reactive lymphoid hyperplasia
- Hodgkin lymphoma

METASTATIC MALIGNANCY[45-48]

Majority of the malignant tumors spread through the lymphatic and therefore the lymph nodes are the most common sites of metastasis. The different types of carcinomas, malignant melanomas, germ cell tumors and certain sarcomas metastasize in the lymph node. For the accurate diagnosis of the type and primary site of the malignancy, one should have the detailed clinical history particularly:

- Chief complaints of the patient
- Past and present history about any malignancy
- Histopathology report of the primary, if available
- Complete physical examination

The detailed information of laboratory investigations may also indicate the possible origin of the primary site. Chest radiographs and CT scan of thorax and abdomen may show space occupying lesion. In addition, simple blood test, urine analysis and biochemical investigations are also important.

Cytology

FNAC of the various metastatic malignancy usually show a polymorphic population of cells consisting of reactive lymphoid cells and discrete or clusters of foreign cells. Monotonous

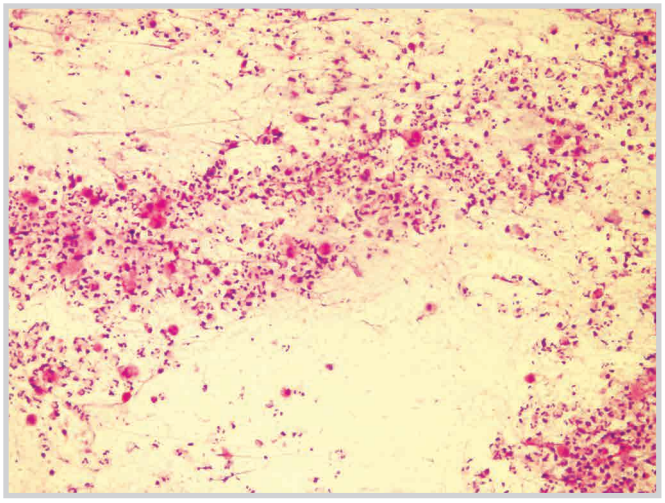


Fig. 28.20: Many discrete malignant squamous cells along with polymorphs in a metastatic squamous cell carcinoma (H & E × MP)

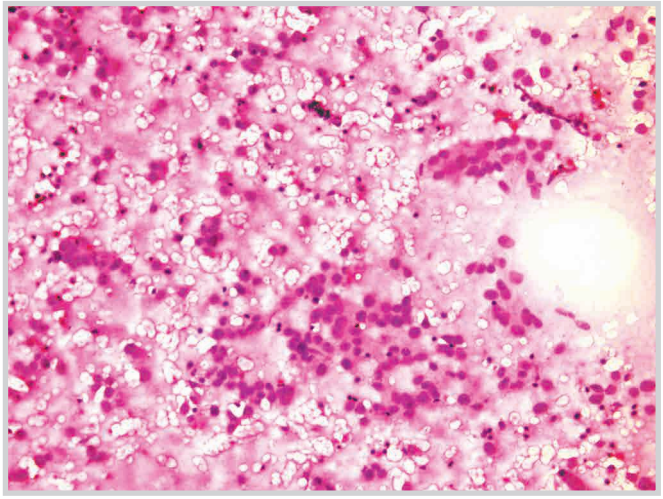


Fig. 28.22: Metastatic adenocarcinoma of prostate in inguinal lymph node (MGG × MP)

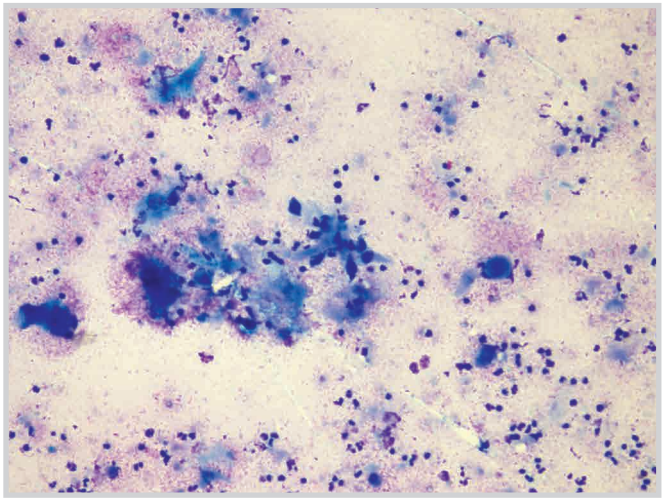


Fig. 28.21: Polyhedral mature-looking squamous cells with enlarged nuclei in metastatic squamous cell carcinoma (MGG × HP)

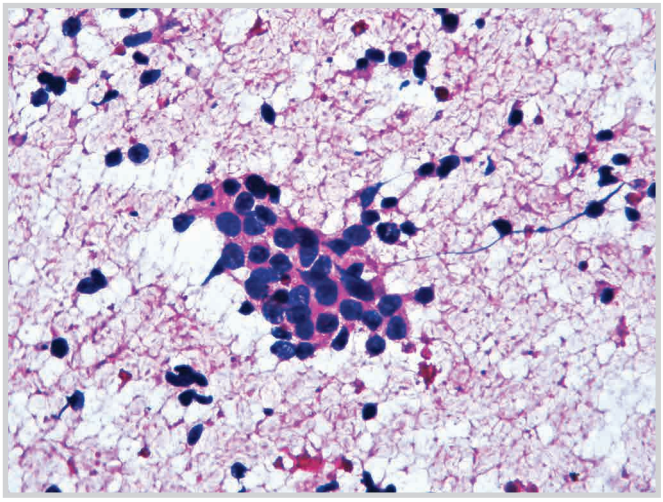


Fig. 28.23: Metastatic small cell carcinoma from lung in supraclavicular lymph node (H & E × HP)

population of malignant cells is seen in case of complete effacement of the lymph node by malignancies.

Squamous Cell Carcinoma (Figs 28.20 and 28.21)

FNAC of the metastatic squamous cell carcinoma in lymph node often show cystic degeneration and the aspirate yield turbid fluid. The cyst should always be re-aspirated to have more cells. The smears show polyhedral squamoid cells with moderate cytoplasm and enlarged hyperchromatic, pleomorphic nuclei. Papaniolaou's stained smear shows orangeophilic cytoplasm indicating intracellular keratin. Many tadpole and fiber cells are also seen. At times, there may be only mature-looking squamous cells in the smear and therefore a definite diagnosis of metastatic squamous cell carcinoma may be difficult. Histopathology should be done in such cases.

Adenocarcinoma (Fig. 28.22)

FNAC smears of adenocarcinoma show loose clusters and discrete cells along with glandular arrangement of malignant cells. The individual cells show moderate amount of vacuolated cytoplasm and central to eccentric nuclei with prominent nucleoli. In addition, the background of the smear may show mucinous material.

Small Cell Carcinoma (Fig. 28.23)

The smears show predominantly discrete cells and occasional small loose clusters of cells. The cells are small with scanty cytoplasm having round minimally pleomorphic hyperchromatic nuclei. Nuclear chromatin is condensed and nucleoli are indistinct to absent. Nuclear molding is the characteristic feature of small cell carcinoma.

Germ Cell Tumor (Fig. 28.24)

Testicular or ovarian germ cell tumor often metastasizes in the para-aortic or mediastinal lymph node. The smear shows foamy vacuolated background and tigroid appearance. The cells are predominantly discrete round having central large nuclei. Nuclear chromatin is fine with single prominent nucleoli. Individual cell morphology along with typical background indicates the possibility of metastatic dysgerminoma or seminoma. Cell of endodermal sinus tumor are severely pleomorphic with large prominent nucleoli.

Sarcoma

Sarcomas may also metastasize in the lymph node and 2–5% of patients with soft-tissue sarcomas develop nodal metastases at any point in the course of their disease.[49]

The common sarcomas that may be seen in the lymph node aspirates are:[48]

- Embryonal rhabdomyosarcoma
- Synovial sarcoma
- Leiomyosarcoma
- Fibrosarcoma
- Malignant fibrous histiocytoma

The cytomorphology of the metastatic sarcoma depends on the morphology of the primary malignancy. Without the proper clinical history and/or knowledge of diagnosis of the primary tumor, it is difficult to diagnose the cases of metastatic rhabdomyosarcoma. The differential diagnosis includes NHL, undifferentiated carcinoma, or neuroblastoma. Immunohistochemistry particularly vimentin and desmin may be needed in the diagnosis of metastatic rhabdomyosarcoma.

Metastatic spindle cell sarcoma is relatively easy to diagnosis. However, the exact subtyping may be difficult or impossible in FNAC. The differential diagnosis of metastatic sarcoma with spindle-cell morphology includes spindle-cell carcinoma and melanoma.

Others

Various other malignancies such as melanoma, carcinoid, renal cell carcinoma, etc. may also metastasize in the lymph node (**Figs 28.25** and, **28.26A** and **B**). Cytological features and thorough clinical history are helpful in diagnosis. In difficult situation, immunocytochemistry may also help.

Immunocytochemistry

Immunocytochemistry is of great help in identifying various types of malignancy particularly poorly differentiated occult malignancies (**Table 28.1**).

Squamous cell carcinoma strongly expresses the cytokeratin (CK) 5, CK14 and CK17 as well as CK6 and CK16. It is important to

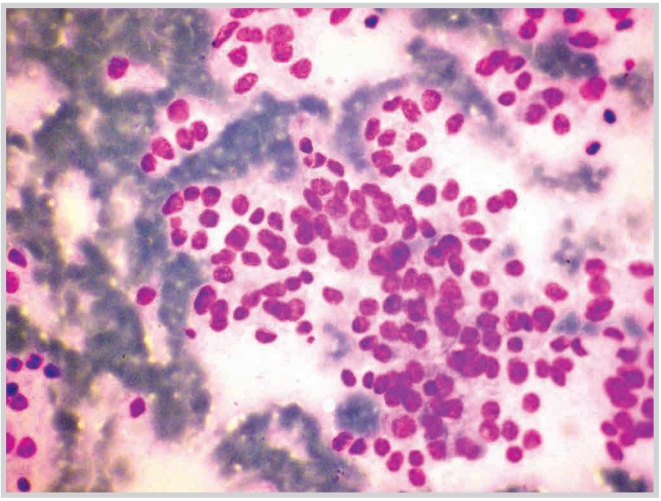


Fig. 28.25: Metastatic carcinoid in a periportal lymph node. Note the discrete round monomorphic cells with scanty cytoplasm (MGG × MP)

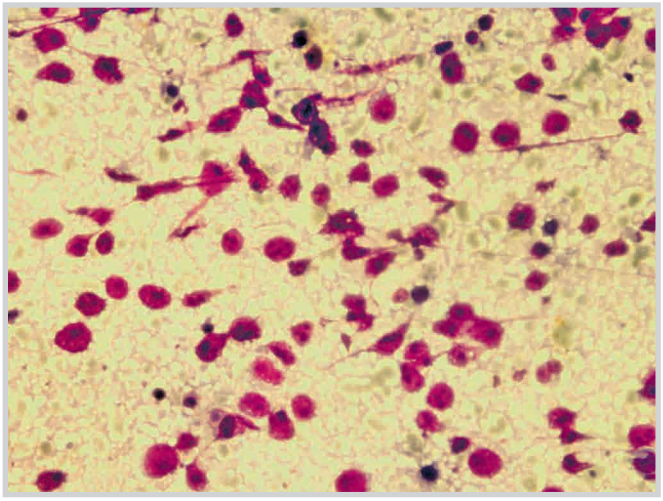


Fig. 28.24: Metastatic seminoma in a para-aortic lymph node. Note the typical trigoid background (MGG × MP)

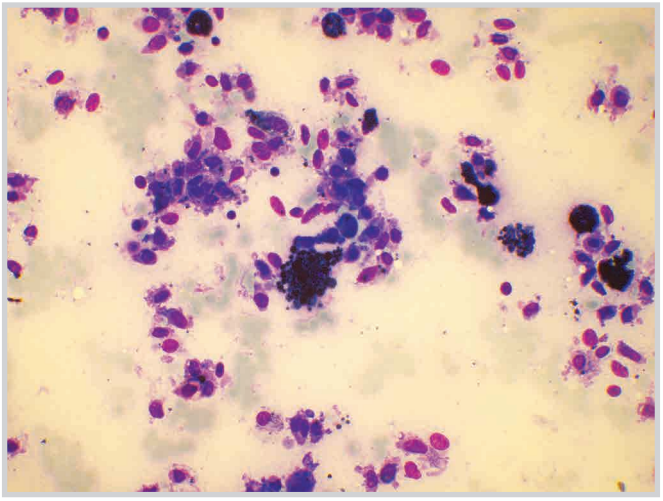


Fig. 28.26A: Metastatic malignant melanoma in lymph node showing dark black melanin pigment within the malignant cells (MGG × MP)

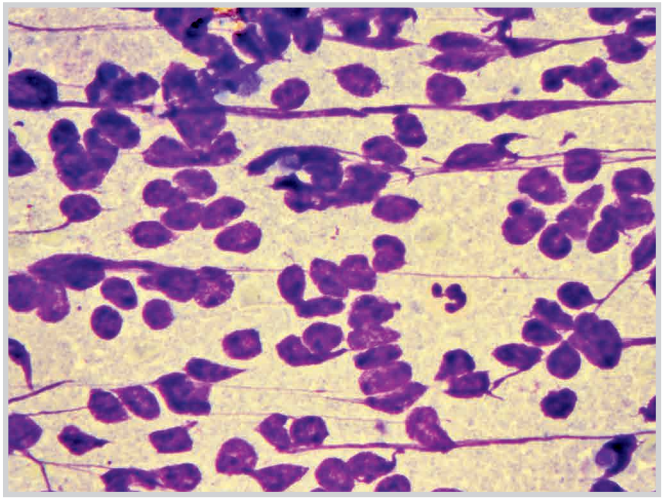


Fig. 28.26B: Metastatic medulloblastoma: Hyperchromatic small round nuclei (MGG × HP)

TABLE 28.1: Immunocytochemistry of different categories of malignancies

Immunocytochemistry (antibody)	*Tumor type*
Keratin and EMA	Epithelial tumors
Vimentin	Mesenchymal tumor
Desmin	Myogenic tumor
CD45 (Leukocyte common antigen)	NHL
Mic-2	PNET
NB-40	Neuroblastoma
HMB 45	Malignant melanoma

Abbreviations: EMA, epithelial membrane antigen; NB-40, nuclear bodies 40; HMB 45, human melanoma black; NHL, non-Hodgkin's lymphoma; PNET, primitive neuroectodermal tumor

remember that the demonstration of CK5 and CK6 expression is particularly helpful to distinguish a poorly-differentiated squamous cell carcinoma from a poorly-differentiated adenocarcinoma.[50, 51]

The detection of primary location of adenocarcinoma is possible by dual use of CK7 and CK20 on FNAC sample (**Table 28.2**). Colorectal adenocarcinomas exhibit a CK7–/CK20+ expression in majority of the cases. Ovary and endometrial carcinomas are always positive for CK7 and negative for CK20. In addition, they are also positive for CK8, CK18 and CK19. Both ductal and lobular carcinomas of breast are positive for CK7, CK8, CK18 and CK19 and negative for CK20, CK5/6 and CK14. Therefore, CK7+, CK20– along with estrogen receptor (ER) and progesterone receptor (PR) positivity are helpful markers to differentiate metastatic breast carcinoma from other adenocarcinomas.[52,53]

In addition to CK, different other antibodies may also be helpful in identifying the exact type of metastatic malignancy such as prostate specific antigen for metastatic prostatic carcinoma, placental alkaline phosphatase (PLAP) for metastatic seminoma, alpha fetoprotein for endodermal sinus tumor, etc.

LYMPHOMAS

Lymphomas are the neoplasm of lymphoid origin. During the process of the development of B or T cells, NHL may origin at any point. The malignant lymphoid cells usually carry the various diagnostic immunological markers of their parental origin. FNAC is one of the most popular and the first line investigations in the diagnosis of lymphomas. The cytopathologist may help in:

- Establishing the primary diagnosis of lymphomas
- Differentiating NHL from HL
- Sub-classification of NHL
- Staging of lymphomas
- Diagnosis of recurrent lymphomas

Lymphoma Classification

Lymphoma has been repeatedly classified by various workers. Each classification has some specific advantages and disadvantages. The changing terminology of NHL reflects the classifier bias and also application of newer technologies.

TABLE 28.2: Cytokeratin expression in different malignancies

Malignancy	*Cytokeratin expression*			
	CK7	*CK20*	*CK5*	*CK19*
Colorectal carcinoma	–	+	–	+
Adenocarcinoma stomach	+	Focal +	–	+
Ovarian adenocarcinoma(non- mucinous)	+	–	–	+
Transitional cell carcinoma	+	+ Focal	–	+
Adenocarcinoma of lung	+	–	–	+
Squamous cell carcinoma of lung	–	–	+	Occasional +
Small cell carcinoma of lung	–	–	–	Focal+
Invasive carcinoma of breast	+	–	+ in basal type	+
Renal cell carcinoma, clear cell	–	–	–	Focal+

Abbreviation: CK, cytokeratin

Working formulation of classification of lymphoma was widely one of the popular classifications of lymphoma.[54] The classification was based on the dominant cytologic cell type observed under the microscope and the presence or absence of "follicles" or nodules. Lymphomas with follicular or nodular pattern behave better than the cases without any follicular or nodular pattern. The application of working formulation was a challenge to the cytopathologists because on FNAC smears it was not possible to comment on follicular pattern as this is purely a histopathological finding. However, there were various attempts to apply the working formulation on FNAC with varying success.[55, 56]

In the year 1994, Revised European American Lymphoma (REAL) classification, was proposed by the International Lymphoma Study Group.[57] Within a couple of years, this was modified by the World Health Organization (WHO) group.[58] This WHO classification of lymphoma is mainly based on the cell lineage of the lymphoid cells and the stage of maturation of the presumed normal counterparts (**Table 28.3**).

This classification is relatively easily applicable on the FNAC smear. For the implementation of WHO lymphoma classification one should have the following information:

- Detailed cytomorphology of the lymphoid cells
- Immunophenotype of the cells
- Molecular genetic markers and cell proliferation markers (preferable).

The different types of NHL have their unique cytomorphology and immunophenotype expression.[59-64]

B Cell Small Lymphocytic Lymphoma

B cell small Lymphocytic lymphoma/chronic lymphocytic leukemia (SLL/CLL) is the neoplasm of mature B cells and represents 6% of NHL. This is an indolent, less aggressive and incurable tumor. The majority of the patients are older than 50 years and the median age of the patient is 65 years. Clinically, the patients are mostly asymptomatic. Occasionally, they may have weakness, hemolytic anemia, lymphadenopathy and hepatosplenomegaly. SLL patients also show leukemia-like picture at the time of presentation. Cytomorphological and phenotypic picture of SLL and CLL are indistinguishable.

Cytomorphology (Figs 28.27 to 28.29)

Cytology smear of SLL shows a monomorphic population of small round cells (**Box 28.13**). These cells are slightly larger than mature

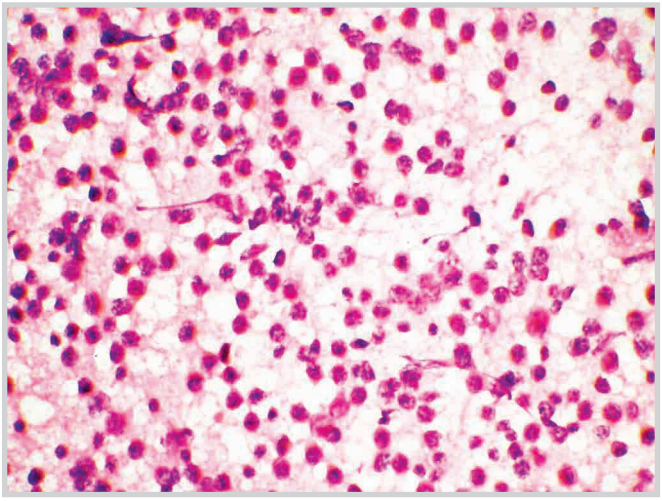


Fig. 28.27: Discrete round monomorphic cells in small lymphocytic lymphoma (SLL) (H & E × LP)

TABLE 28.3: REAL/WHO's classification of non-Hodgkin's lymphoma in brief

B cell non-Hodgkin's lymphoma	*T cell non-Hodgkin's lymphoma*
Precursor B cell neoplasm • Lymphoblastic leukemia/Lymphoma Peripheral B cell neoplasm • B cell CLL/SLL • B cell prolymphocytic leukemia • Lymphoplasmacytic lymphoma • Extranodal marginal zone lymphoma, of mucosa associated lymphoid tissue (MALT lymphoma). • Nodal marginal zone B-cell lymphoma • Mantle cell lymphoma • Follicular lymphoma • Diffuse large B cell lymphoma • Burkitt's lymphoma • Mediastinal large B cell lymphoma • Plasma cell myeloma • Plasmacytoma	Precursor T cell neoplasm • Lymphoblastic leukemia/lymphoma Peripheral T cell neoplasm • T cell prolymphocytic leukemia • T cell large granular lymphocytic (LGL) leukemia • NK cell leukemia • Adult T-cell leukemia/lymphoma • Angioimmunoblastic T-cell lymphoma • Anaplastic large cell lymphoma, T/null-cell • Mycosis fungoides • Sezary syndrome • Hepatosplenic T cell lymphoma

Abbreviations: REAL, Revised European American Lymphoma; WHO, World Health Organization; CLL, chronic lymphocytic leukemia; SLL, small lymphocytic lymphoma; NK, natural killer

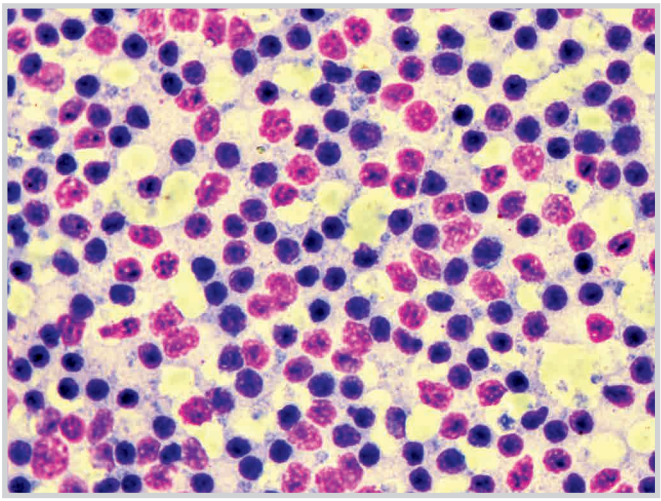


Fig. 28.28: Fine needle aspiration cytology (FNAC) smear of small lymphocytic lymphoma (SLL) showing round cells with scanty cytoplasm. Nuclei show condensed clumped chromatin (MGG × HP)

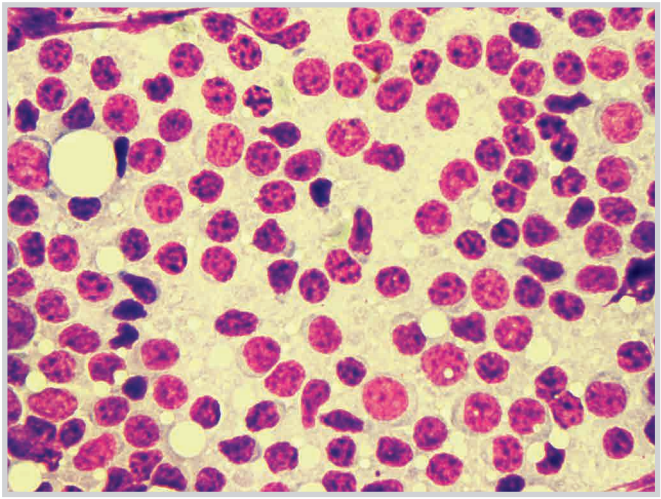


Fig. 28.29: Higher magnification showing typical clumped chromatin in nuclei of Small lymphocytic lymphoma (SLL) case (MGG × OI)

BOX 28.13 **Small lymphocytic lymphoma/Chronic lymphocytic leukemia**

- Small lymphocytes
- Small round regular nucleus
- Regular nuclear membrane
- Coarsely clumped chromatin
- No nucleoli
- Immunophenotype:
- Positive for: CD5, CD23, CD19, CD20; Co-expression of CD 5 and CD23
- Negative for: CD10

lymphocytes. The individual cells have scanty barely visible thin rim of cytoplasm. Nuclei of the cells are round with minimal pleomorphism and almost regular nuclear margin. Nuclear chromatin is coarsely clumped with absent to inconspicuous nucleoli. Mitotic activity is very low. Scanty to moderate number of prolymphocytes and paraimmunoblasts are also seen in FNAC smear. The prolymphocytes are medium-sized cells, larger than the small lymphocytes, with dispersed chromatin and small nucleoli. The paraimmunoblasts are medium to large with scanty basophilic cytoplasm and vesicular nuclei having central prominent nucleoli. These cells are seen in aggregates in the proliferation center and may not be easily distinguishable from each other. The presence of more than 10% prolymphocytes indicates more aggressive course of the disease. About 3.5% cases of SLL may undergo transformation to high grade NHL in course of time. The most common type of transformation is diffuse large B cell lymphoma (DLBCL). The aspirates of these cases show more than 50% large lymphoma cells that have scanty-to-moderate amounts of cytoplasm with 1 or 2 prominent nucleoli. In addition, the smears show increased numbers of mitotic figures, apoptotic bodies, the presence of necrosis, and a myxoid and dirty background. Ki 67 index (an antigen) more than 30% is another helpful indicator of transformation to high grade NHL.[65]

Immunophenotype

CLL/SLL cells are typically positive for CD5+, CD23+, CD19 + and CD20+ (weak) and negative for CD10 and FMC-7. The cells show co-expression of CD5 and CD23.

Genetics: Trisomy 12 or 13q present in some cases.

Mantle Cell Lymphoma

Mantle cell lymphoma (**Box 28.14**) is an aggressive B cell neoplasm that represents 3–10% of NHL. The disease occurs in elderly patient above 60 years with a higher male predominance. The patients of MCL usually present in higher stage with generalized lymphadenopathy and hepatosplenomegaly. The peripheral blood may show involvement in one-fourth of the case at the time of presentation. MCL commonly involves lymph node and spleen. The most common extranodal site of involvement of MCL is gastrointestinal tract. MCL has an aggressive course with multiple relapses and progression of the disease. The vast majority of the patients are not cured. The median survival of this NHL is only 3–5 years. Morphologically, MCL is indistinguishable from SLL.

Cytomorphology (Fig. 28.30)

Cytology smears show small lymphoid cells with scanty thin rim of cytoplasm. Nucleus is monomorphic with irregular margin, dispersed fine chromatin and inconspicuous nucleoli. There is absence of prolymphocytes or paraimmunoblasts. The lymphoid cells may be in aggregation in about one-third of cases. In the blastic variant of MCL, the cells are of intermediate to large and the nuclei are approximately four times larger than a small, mature lymphocyte nucleus with irregular contours and fine, evenly distributed chromatin. Mitotic activity is high in blastic variant of MCL. This type of MCL has poorer prognosis.[66,67]

Immunophenotype

- Positive: CD20, CD5, FMC 7, Cyclin D1
- Negative: CD23
- *Genetics:* t(11:14); BCL1
- Gene rearrangement

BOX 28.14 Mantle cell lymphoma

- Small to medium sized cells
- Irregular nuclear contour
- Fine nuclear chromatin
- Inconspicuous nucleoli

Immunophenotype: CD5+, CD10-, CD19 +, CD20+, CD23-, Cyclin D1 positive

BOX 28.15 Follicular lymphoma

- A mixture of cleaved and non-cleaved small and large cells
- Small cells with cleaved nuclei
  - Coarse chromatin
  - Absent nucleoli
- Large non-cleaved cells
  - Round regular nuclei
  - Fine nuclear chromatin and multiple nucleoli

Immunophenotype: Surface Ig +, CD5–, CD10+, CD19+, CD20 + , BCL2+

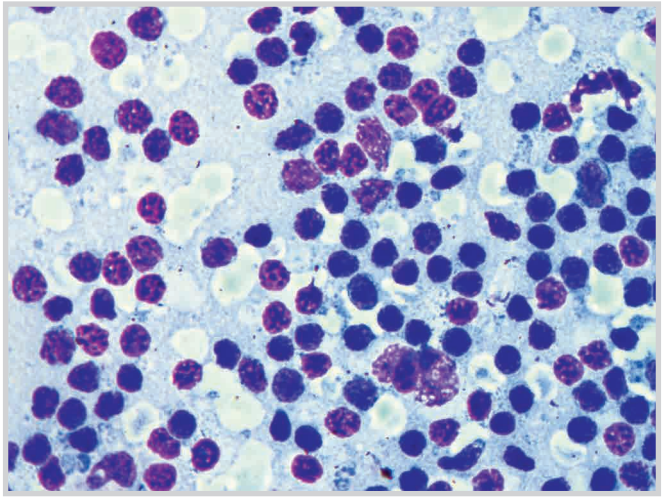


Fig. 28.30: Fine needle aspiration cytology (FNAC) smear of mantle cell lymphoma showing small lymphoid cells with scanty thin rim of cytoplasm and monomorphic nuclei with dispersed fine chromatin and inconspicuous nucleoli (MGG × OI)

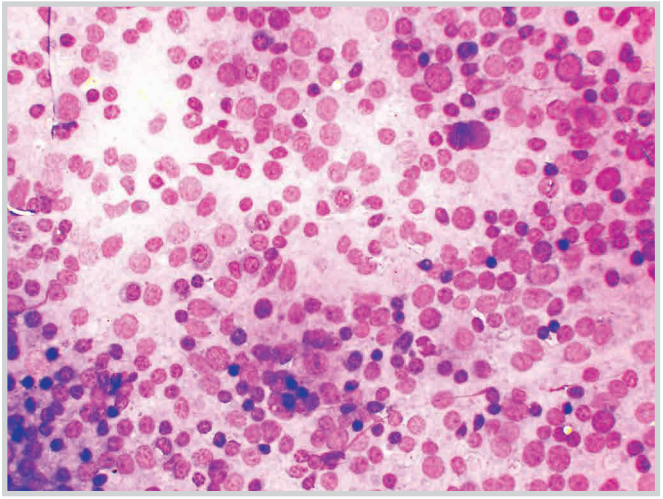


Fig. 28.31A: Follicular lymphoma: Mixed small and large lymphoid cells in follicular lymphoma (MGG × HP)

Follicular Lymphoma

Follicular lymphoma (**Box 28.15**) is a neoplasm of follicular center B cells. It is the commonest variety of NHL and represents 20–35% NHL. FL usually occurs in elderly patient with a median age 59 years. The most patients present with generalized lymphadenopathy involvement of spleen and also bone marrow at the time of diagnosis.[68] This is an indolent lymphoma and 5 years survival rate is high. However, the patients are refractory to treatment.

Cytomorphology (Figs 28.31A to C)

WHO recommends three grades system of FL on histology:

Grade 1: Predominantly centrocytes: 0–5 centroblasts/high power field

Grade 2: Mixture of centrocytes and centroblasts: 6–15 centroblasts/high power field

Grade 3: Predominantly centroblasts: More than 15 centroblasts/high power field

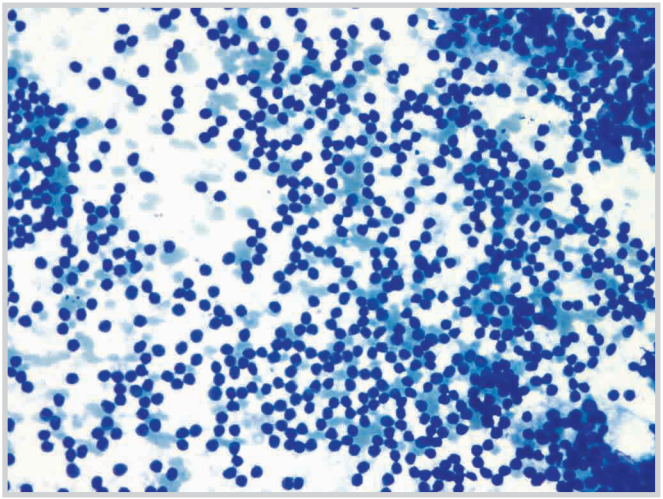


Fig. 28.31B: Follicular lymphoma: Discrete small cleaved cells in an intra-abdominal lymph node (MGG × HP)

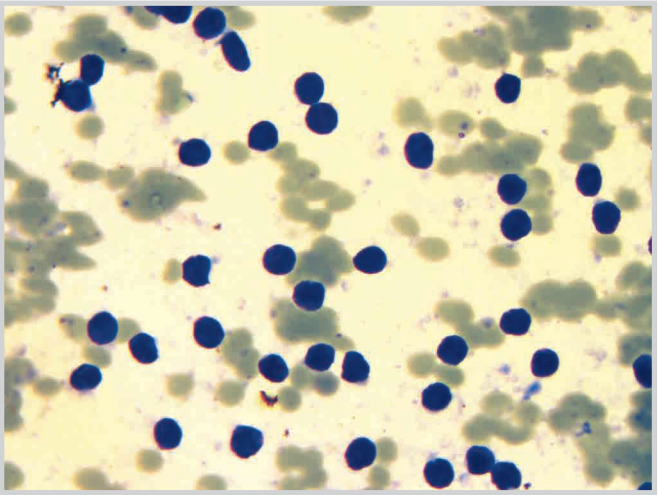


Fig. 28.31C: Follicular lymphoma: Higher magnification of the same (MGG × OI)

Grade 3a: Centrocytes present
Grade 3b: Solid sheets of centroblasts only

The cells are morphologically neoplastic equivalent of the normal germinal cell. FNAC smears show a monomorphic population of cell consisting of centrocytes and mixed with cleaved and non-cleaved centroblasts-like cells. The small cleaved cells are small in size with cleaved nuclei having coarse chromatin and absent nucleoli. They resemble centrocytes. The centroblast-like cells have large regular nuclei having fine nuclear chromatin and multiple nucleoli. The number of these large cells is between 20% and 50%. In addition, the smear may also show aggregation of dendritic histiocytes in 33% cases.[69] On cytology smear, it is almost impossible to say about the follicular pattern of the cells. The grade of FL on cytology smear is also subjective as the count of centrocytes and centroblasts is subjective in a smear. The distinction between a grade 3 FL and DLBCL is also not possible in FNAC smears.

Immunophenotype

- Positive: CD19, CD20, CD10, BCL2
- Negative: CD5
- *Genetics:* t(14:18) and BCL2 gene rearrangement

Lymphoplasmacytic Lymphoma

Lymphoplasmacytic lymphoma (LPL) (**Box 28.16**) is a relatively uncommon lymphoma and accounts for 1.5% of all nodal lymphoma. This is a neoplasm of the small B lymphocytes, plasma cells and plasmacytoid cells. LPL usually occurs in the elderly patients. The tumor commonly involves lymph node, spleen and bone marrow along with peripheral blood. The majority of the patients show Immunoglobulin M (I_GM) monoclonal protein in the serum. If the serum level of IgM protein exceeds 3 gm/100 ml then the disease is labeled as Waldenstrom's macrogloblinemia. The patients with high I_GM may have hyperviscosity syndrome. LPL has an indolent course and only a small fraction of the lymphoma may transform to DLBCL.

BOX 28.16 Lymphoplasmacytic lymphoma

- Lymphocytes
- Plasma cells
- Plasmacytoid cells
- Occasionally immunoblasts, epithelioid histiocytes and mast cells
- Immunocytochemistry:

Positive: Cytoplasmic immunoglobulin, CD I9, CD20, CD22, CD 79a, CD38
Negative: CD5, CD23, CD10

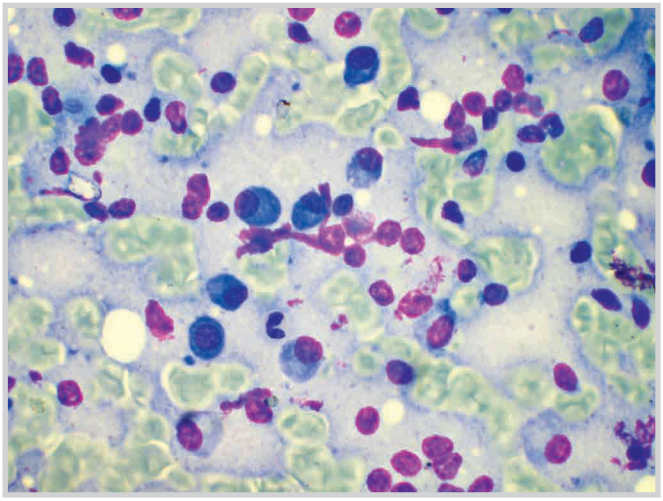


Fig. 28.32: Lymphoplasmacytoid lymphoma showing plasmacytoid cells and small mature looking lymphoid cells (MGG × HP)

Cytology (Fig. 28.32)

Smears show a mixture of lymphocytes, plasma cells and plasmacytoid cells. In some cases, intracytoplasmic *periodic acid Schiff* (PAS) positive inclusion bodies (Dutcher bodies) are also seen. Rarely immunoblasts, epithelioid histiocytes and mast cells are present in the smear.[68]

The LPL is the disease of exclusion as various other lymphomas such as FL, MCL and SLL also simulate similar morphological features.

Immunocytochemistry

Positive: Cytoplasmic immunoglobulin, COI9, CD20, CD22, CD79a, CD38.

Negative: CD5, 23, CD10.

Marginal Zone Lymphoma

Marginal zone lymphoma (MZL) is relatively uncommon low-grade B cell lymphoma (**Box 28.17**). MZL may be nodal and extra-nodal MZL. Extra-nodal marginal zone lymphoma is commonly known as mucosa-associated lymphoid tissue (MALT) lymphoma and represents 7–8 % of all lymphomas. The extra-nodal MZL occurs in various body sites such as stomach, eye, thyroid,

BOX 28.17 Marginal zone/MALT lymphoma

- Heterogeneous population
- Small centrocyte like cells
- Plasma cells
- Plasmacytoid cells
- Monocytoid B cells with pale cytoplasm

Immunophenotype: Surface Ig+, CD5-, CD10-, CD19 +, CD 20+, CD23–

Abbreviation: MALT, mucosa-associated lymphoid tissue

salivary gland, lung and skin. The stomach is the most frequently involved area of MALT lymphoma. In many cases of MALT lymphoma, a strong association with autoimmune diseases is noted such as Hashimoto's thyroiditis in thyroid lymphoma, Sjögren's syndrome in the salivary gland and *Helicobacter pylori* infection induced gastritis in stomach lymphomas.

Cytomorphology

Cytology smear of MZL shows a heterogeneous population of cells consisting of small to medium-sized lymphoid cells with irregular nuclear contour, plasma cells, plasmacytoid cells and variable numbers of monocytoid cells. The small cells have convoluted nuclei with condensed chromatin and inconspicuous nucleoli. The cells resemble centrocytes. The monocytoid cells have moderate amount of pale staining cytoplasm with centrally placed convoluted nuclei having dispersed chromatin and inconspicuous nucleoli. Tingible body macrophages and lymphohistiocytic aggregations are also seen in the smear.

Immunophenotype

Surface Ig+, CD5-, CD10-, CD 19+, CD20+, CD23–.

APPROACH TO SMALL B CELL LYMPHOMAS

On FNAC smears, it is important to differentiate these B cell lymphomas from a RLH. The following features are suggestive of RLH:

- Polymorphic population of lymphoid cells
- Lymphohistiocytic aggregates
- Tingible body macrophages

These features are however not applicable to distinguish NHL and all cases of RLH. In case of partially involved NHL, there may be admixture of polymorphic population of lymphoid cells. Unlike other NHL, MZL may show a polymorphic population of lymphoid cells consisting of centrocytes, centroblasts, plasma cells and plasmacytoid cells. Dendritic-lymphocytic aggregates and tingible-body macrophages may be seen in FL. Therefore, to resolve the issue flow cytometric immunophenotyping and the demonstration of light chain restriction are necessary (**Fig. 28.33**).[1]

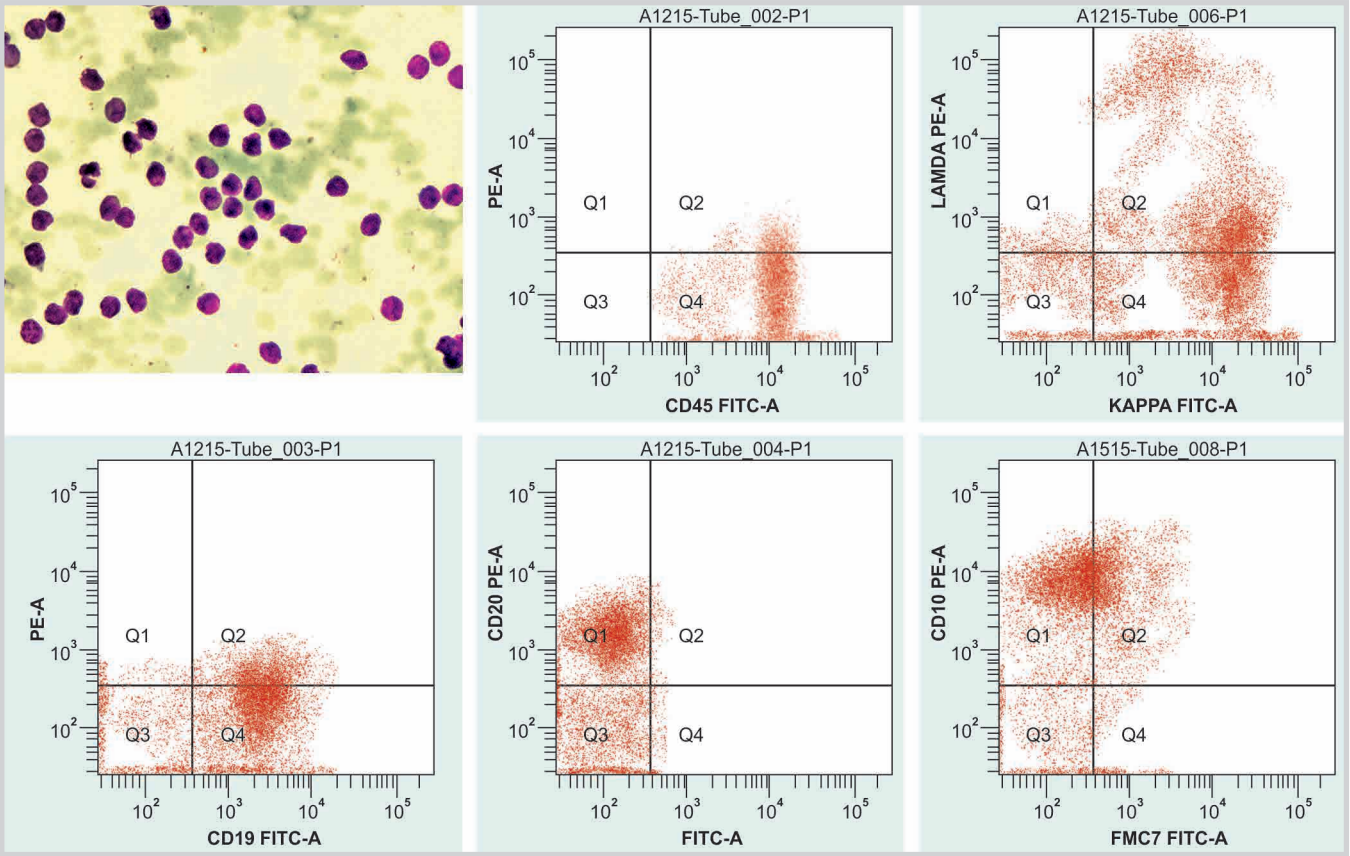


Fig. 28.33: A case of follicular lymphoma in intra abdominal lymph node showing small mature-looking lymphoid cells. Flow cytometric immunophenotyping shows CD45, CD19, CD20 and CD10 positivity. The cells also show light chain restriction (predominant kappa chain expression)

BOX 28.18 Molecular genetics of mature small B cell NHL

- SLL: trisomy 12 or 13q
- MCL: translocation of t(11;14)(q13; q32)
- FL: t(14:18)(q32;q21) translocation

Abbreviations: FL, follicular lymphoma, MCL, mantle cell lymphoma, SLL, small lymphocytic lymphoma

The non-lymphoid malignancies, particularly small cell carcinoma may also simulate NHL in FNAC. The cells of small cell carcinoma show frequent nuclear molding, paranuclear blue bodies and background crushing artifact. In addition, the cells may also be present in tight clusters. These cells are positive for synaptophysin and chromogranin and negative for CD45 (leukocyte common antigen, LCA).

The five common types of mature B cell NHL often poses diagnostic difficulties. **Table 28.4** shows detailed immunophenotyping features to distinguish these lymphomas.

SLL and MCL both are positive for CD 5. However, SLL cases show dual expression of CD 5 and CD 23, whereas MCL shows only CD5 positivity. FL, LPL and MZL all are negative for CD5. Among these three entities, CD 10 positivity is noted only in FL. Cyclin D1 expression is quite specific for MCL (**Flow chart 1**).

In addition, molecular genetics study is also helpful in difficult cases where aberrant expression of *flow cytometry immunophenotyping* (FCI) happens. SLL cases often show trisomy 12 or 13q (**Box 28.18**). The translocation of t(11;14)(q13;q32) is characteristic of MCL. FL shows t(14:18)(q32;q21) translocation and BCl2 gene rearrangement in majority of the cases.[1]

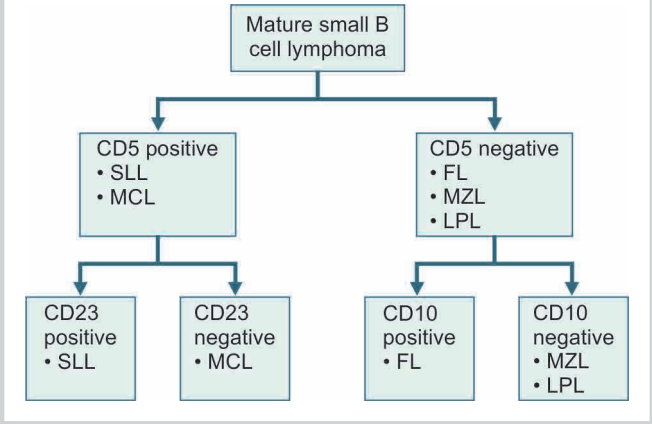
LYMPHOMAS OF LARGE CELLS

Diffuse Large B Cell Lymphoma

Diffuse large B cell lymphoma is one of the frequently encountered NHL and represents 30–40% of adult NHL. The elderly patients are usually commonly affected; however, the children are not exempted from DLBCL. There are both nodal and extra-nodal involvement of DLBCL and about 40% patients of DLBCL show extra-nodal involvement and out which, stomach is the commonest site of involvement.[68]

This lymphoma commonly develops de novo or may represent the transformation or progression of other less aggressive lymphomas such as SLL, FL and MALT/MZL lymphomas and nodular lymphocytic predominant HL.

Flow chart 1: Mature small B cell lymphoma



Abbreviations: MCL, mantle cell lymphoma; SLL, small lymphocytic lymphoma; FL, follicular lymphoma; MZL, marginal zone lymphoma; LPL, lymphoplasmacytic lymphoma

Cytomorphology (Figs 28.34 and, 28.35A and B)

On histology section, DLBCL shows discrete large lymphoid cells with different morphologic variants:

- Centroblastic
- Immunoblastic
- T-cell or histiocyte rich
- Anaplastic large B-cell

The recognition of these morphologic variants are too some extent subjective and may not have any further prognostic value.[58] FNAC smears show monomorphic large cells two to three times of mature lymphocytes (**Box 28.19**). The individual

TABLE 28.4: Immunophenotype of small mature B cell lymphomas

Lymphomas	*Antigen*					
	CD10	*CD5*	*CD23*	*Cyclin D1*	*CD20*	*CD38*
SLL	Negative	Positive	Positive	Negative	Weak positive	-
MCL	Negative	Positive	Negative	Positive	Positive	-
FL	Positive	Negative	Variable positivity	Negative	Positive	-
LPL	-	-	-	-	+	+
MZL	-	-	-	-	+	-

Abbreviations: SLL, B cell small lymphocytic lymphoma; MCL, mantle cell lymphoma, FL, follicular lymphoma; LPL, lymphoplasmacytic lymphoma; MZL, marginal zone lymphoma

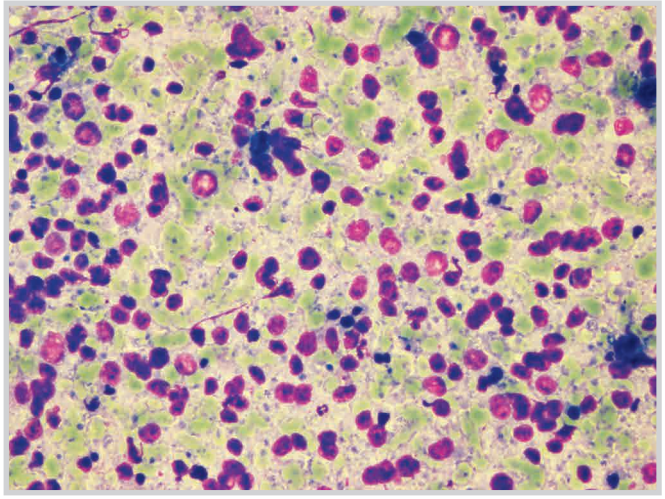


Fig. 28.34A: Discrete immature lymphoid cells in diffuse large B cell lymphoma (DLBCL) (MGG × MP)

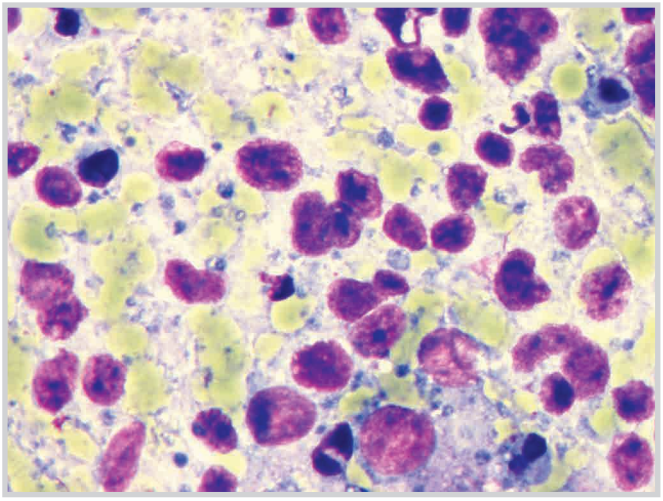


Fig. 28.34B: Higher magnification showing large cells with scanty cytoplasm and occasional prominent nucleoli (MGG × OI)

BOX 28.19 **Diffuse large B cell lymphoma**

- Monomorphic large cells two to three times of mature lymphocytes
- Prominent nucleoli and basophilic cytoplasm
- Variable mitotic figures
- Ki67 index less than 90%

Immunophenotype: Surface Ig +/-, CD5–, CD10+/–, CD19+, CD20+

cells have scanty thin rim of basophilic cytoplasm, round nuclei with fine chromatin and large prominent nucleoli. The cytomorphology depends on the individual morphologic variants of DLBCL.

Centroblastic

Cytology smears of this variant show medium to large cells. These cells are 2–3 times larger than lymphocyte and have scanty basophilic cytoplasm. Nuclei of the cells are round and vesicular with fine chromatin. Each nucleus has 2–3 prominent membrane-bound nucleoli. The cells may be admixed with multilobated cells and immunoblasts.

Immunoblastic

In this variant more than 90% of the cells are immunoblasts. The cells are large with scanty to moderate amount of basophilic cytoplasm. Nuclei show central large nucleoli with peripheral clearing of chromatin. The smear shows less than 10% centroblasts. Occasionally there may be plasmacytoid differentiation of the immunoblasts and may pose difficulty to distinguishing with plasmablastic multiple myeloma.

T Cell/Histiocytes Rich (Figs 28.35A and B)

The smear is composed of more than 90% reactive T cells and less than 10% neoplastic large B cell that resemble immunoblasts, centroblasts or RS cell. In addition, there may be variable numbers of non-neoplastic histiocytes. These histiocytes often give epithelioid cell like appearance and may simulate epithelioid cell granuloma. This NHL may be mistakenly diagnosed as granulomatous reaction in FNAC.

Anaplastic

In this variant, the smear is composed of large round to oval cells with bizarre pleomorphic nuclei.

- This variant has no relation with anaplastic large cell lymphoma.

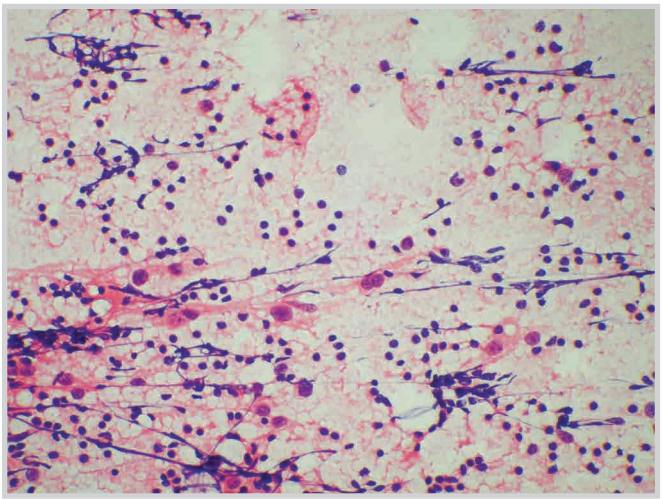


Fig. 28.35A: Discrete occasional atypical lymphoid cells in the reactive lymphoid cell background in T cell-rich B cell lymphoma (MGG × MP)

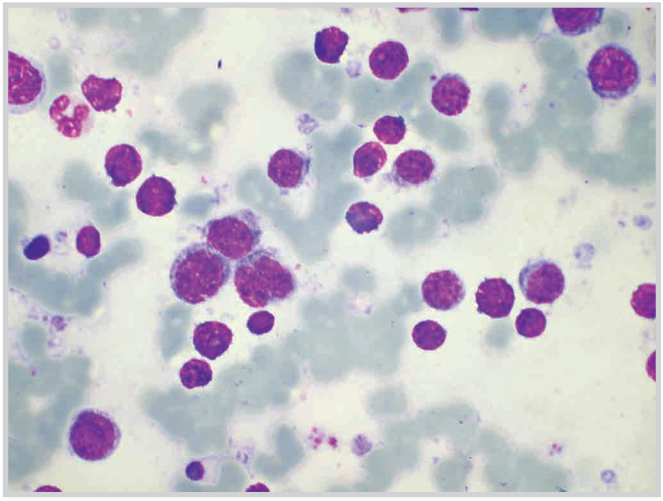


Fig. 28.35B: Higher magnification showing better morphology of the neoplastic lymphoid cells (MGG × OI)

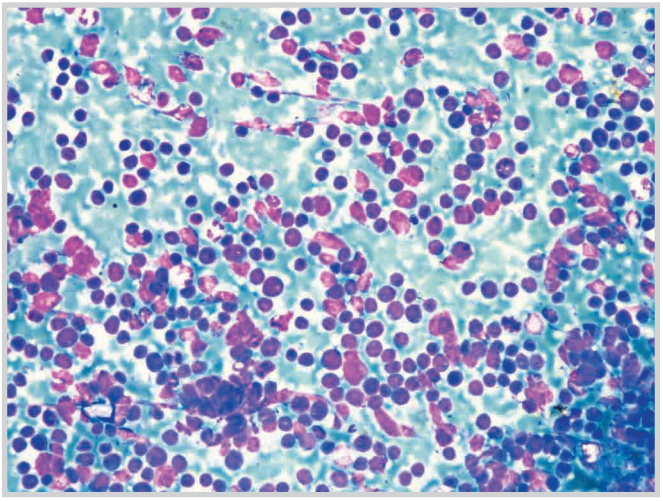


Fig. 28.36: Fine needle aspiration cytology (FNAC) smear of lymphoblastic lymphoma showing discrete immature lymphoid cells (MGG × MP)

Immunophenotype

- Positive: CD19, CD20 and CD79a, surface and cytoplasmic Ig. DLBCL variably expresses CD10
- Negative: CD5

Flow Cytometry Immunophenotyping

Flow cytometry immunophenotyping of DLBCL is often difficult as the cells are fragile besides that the large cells may not come into the usual lymphoid gated population.

Genetics: t(14:18) in 30% cases.

B Lymphoblastic Lymphoma

Precursor lymphoblastic lymphoma (LBL) is also called as acute lymphoblastic leukemia. LBL is usually seen in children and young adults.

Cytomorphology (Figs 28.36 and 28.37)

The tumor shows a monomorphic population of small to medium sized cells immature lymphoid cells. The cells have scanty basophilic cytoplasm and round nuclei (**Box 28.20**). Nuclear chromatin is condensed with inconspicuous nucleoli. Occasional cells may have moderate amount of vacuolated cytoplasm and prominent nucleoli.

Immunophenotype

LBL is characteristically positive for terminal deoxynucleotidyl transferase (Tdt). The cells are positive for CD10 and CD19.

Burkitt's Lymphoma

Burkitt's lymphoma is a highly aggressive B cell NHL with frequent extra-nodal involvement and is characterized by

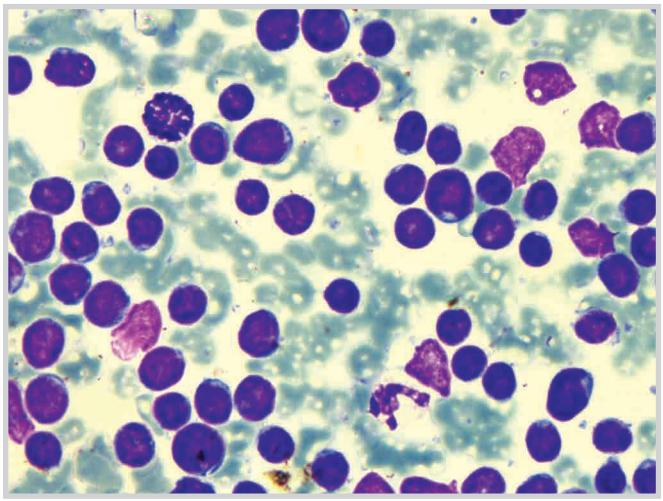


Fig. 28.37: Higher magnification of lymphoblastic lymphoma showing detailed nuclear morphology. The cells have scanty cytoplasm, round nuclei with homogenous condensed chromatin and inconspicuous nucleoli (MGG × OI)

BOX 28.20 Lymphoblastic lymphoma

- Small to medium sized cells
- Scanty basophilic cytoplasm
- Condensed nuclear chromatin and small inconspicuous nucleoli
- Nuclear contour irregularity may or may not be present

Immunophenotype: Tdt (deoxynucleotidyl transferase)+, CD10+, CD19+

cytogenetic translocation that involves the c-myc oncogene (**Box 28.21**). There are three clinical variants of BL:

1. *Endemic:* It is commonly noted in Africa and also to a lesser extent in New Guinea and South America. This is the most common malignancy in childhood in this zone. The endemic type of BL usually presents with jaw swelling and abdominal mass.

BOX 28.21 Burkitt's lymphoma

- Medium sized cells
- Moderate amount of deep basophilic vacuolated cytoplasm
- Coarse nuclear chromatin with multiple prominent nucleoli
- Starry sky appearance
- High mitotic rate
- High apoptotic rate

Immunophenotype: Surface Ig+, CD5–, CD23–, CD10+, CD19+, CD20 +, Ki67 index is more than 85%
Genetics: t(8:14), t(8:22). Rearrangement of c-myc gene

2. *Sporadic:* This variant is noted throughout the world. The children and young adults are commonly affected by sporadic variant of BL. It is often associated with Epstein-Barr virus (EBV) infection and poor socioeconomic condition. The sporadic variant represents 30–50% of all childhood lymphomas.
3. *Immunodeficiency related BL:* This variant is associated with HIV infection and the patients present as the initial manifestation of AIDS.

Cytomorphology (Figs 28.38A and B, and 28.39)

Cytology smear of BL shows a dimorphic population of cells. The dark colored lymphoid cells and pale scattered tingible body macrophages. This gives a starry sky appearance of the smear.[70,71]

The lymphoid cells are medium sized with moderate amount of deep basophilic vacuolated cytoplasm. The nucleus is round with coarse chromatin having two to three variable sized prominent nucleoli. Frequent mitotic activity is also seen. Background of the smear contains many tingible body macrophages.

Immunophenotype

- Positive: Surface Ig, CD10, CD19, CD20
- Negative: CD5, CD23
- Cell proliferation markers: Ki67 index is more than 85%
- Endemic form of BL harbors latent EBV genome

Genetics: t(8:14), t(8:22) and rearrangement of c-MYC gene.

T cell lymphoma

T cell NHL represents 10% of all NHL. It usually occurs in adults.

WHO classified this NHL as precursor T lymphoblastic lymphomas and mature T cell lymphomas.

T Lymphoblastic Lymphoma

It is highly aggressive and usually occurs in young adults. Most of the patients present with palpable neck node along with mediastinal lymphadenopathy that often compresses the superior venal cava causing superior venal cava obstruction.

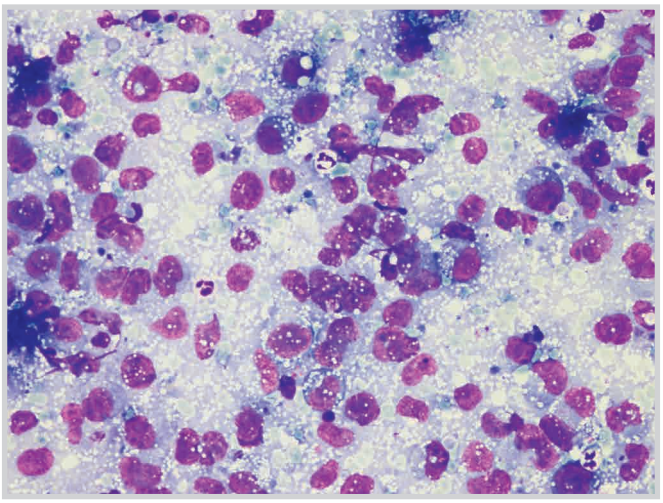


Fig. 28.38A: Burkitt's lymphoma: Discrete lymphoid cells with vacuolated cytoplasm (MGG X MP)

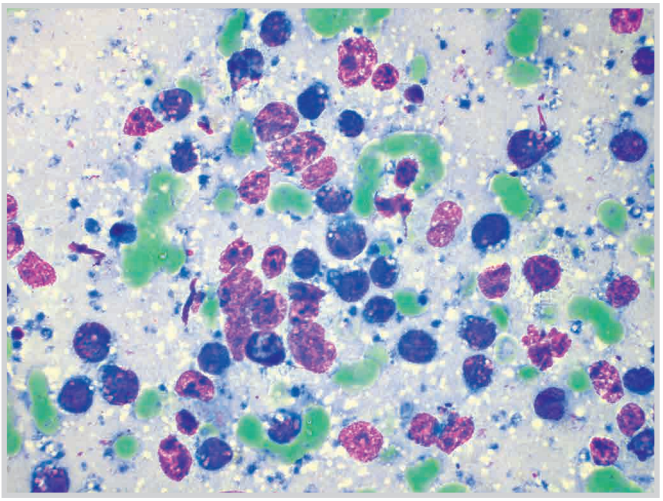


Fig. 28.38B: Burkitt's lymphoma: Lymphoid cells with vacuolated cytoplasm and relatively large nuclei with multiple prominent nucleoli (MGG × HP)

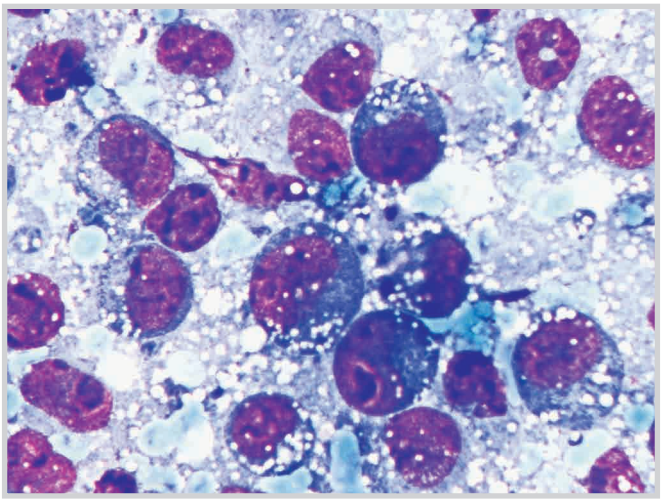


Fig. 28.39: Burkitt's lymphoma: Individual cell morphology is better seen in higher magnification. Enlarged cells with large nuclei having prominent nucleoli (MGG × OI)

FNAC diagnosis of this lymph node and rapid therapy gives a great relief to the patient.

Cytomorphology

FNAC smears show similar morphology as that of B-LBL.

Immunophenotype

TdT +, CD3+, CD7+, CD4+/–, CD8+

Anaplastic Large Cell Lymphoma

Anaplastic large cell lymphoma (ALCL) is a T cell neoplasm and represents 3 % of all adult NHL and about 30% of childhood NHL. The majority of the patients present with higher stage and extra-nodal involvement at the time of first presentation. Systemic symptoms especially fever is also noted.

The diagnostic cytological features are mentioned in **Box 28.22**.

Cytomorphology[72,73] (Figs 28.40A and B to 28.42)

FNAC smears show many large cells and medium sized cells with bi- and multilobated bizarre nuclei, clumped chromatin and prominent nucleoli (**Box 28.22**). The smears show variable numbers of the characteristic "Hallmark" cells. These cells contain abundant amphophilic cytoplasm with central to eccentric large horseshoe-shaped convoluted nuclei and single to multiple prominent nucleoli. Occasionally such kidney-shaped nuclei show a smooth convex surface and mildly irregular convex surface. This gives the nuclei a jellyfish or embryo-like appearance. In addition the smear may show "Doughnut" cells that contain a large pseudonuclear cytoplasmic inclusion due to the deep cytoplasmic invaginations into the nuclei. In addition, there may be multinucleated giant cells with abundant cytoplasm and multiple nuclei in a wreath-like pattern with variable sized single to multiple nucleoli. These cells resemble RS cells.

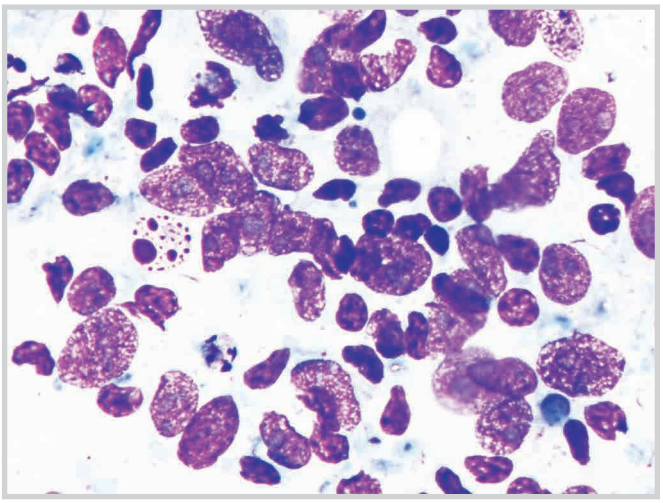


Fig. 28.40B: Anaplastic large cell lymphoma (ALCL). Higher magnification showing better morphology of the cells. Individual cells are large with fine chromatin and prominent nucleoli (MGG × OI)

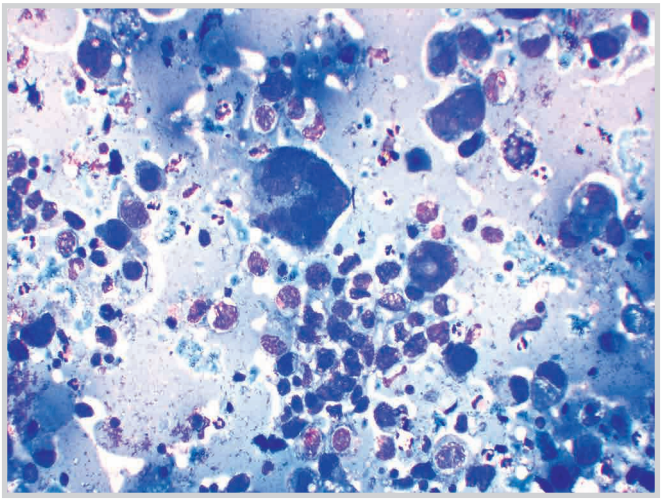


Fig. 28.41: Large doughnut-shaped nuclei in anaplastic large cell lymphoma (ALCL) (MGG × OI)

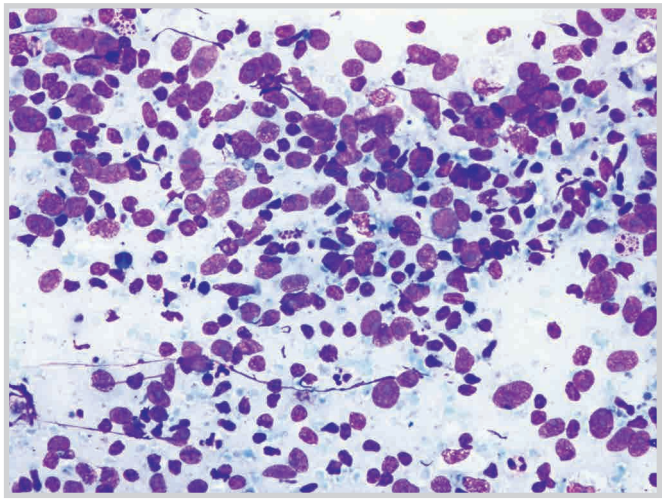


Fig. 28.40A: Anaplastic large cell lymphoma (ALCL). Discrete cells with mild to moderately pleomorphic nuclei (MGG × HP)

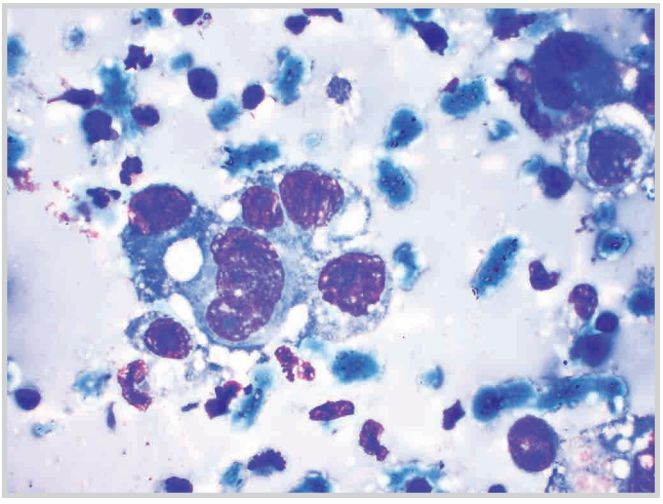


Fig. 28.42: Fine needle aspiration cytology (FNAC) smear of Anaplastic large cell lymphoma (ALCL) showing large cells with moderate amount of cytoplasm. Nuclei have irregular margin, fine chromatin and multiple prominent nucleoli (MGG × OI)

BOX 28.22 Anaplastic large cell lymphoma

- Large cells
- Multilobated nuclei
- "Hallmark" cells with abundant cytoplasm, large horse shoe shaped convoluted nuclei and single to multiple prominent nucleoli
- Doughnut cells
- Multinucleated giant cells with abundant cytoplasm and multiple nuclei in a wreath like pattern
- Plasmacytoid cell with clumped chromatin and prominent nucleoli

Immunophenotype: CD30 (Ki-1) +, EMA+, ALK+

The tumor often contains medium sized cells with moderate cytoplasm and eccentric nuclei giving a "plasmacytoid" appearance.

Immunophenotype

Only two-thirds cases of ALCL are positive for CD 45. As the tumor cells are T cells, so they are positive for CD3 marker. The tumor cells of ALCL are positive for CD30 (Ki-1) and *epithelial membrane antigen* (EMA). Majority of ALCL cases (80%) show membranous or cytoplasmic positivity of anaplastic lymphoma kinase (ALK). The major subset of ALCL shows (ALK) protein that is a useful prognostic marker.

Genetics: t (2:5), causing fusion of ALK and nucleophosmin (NPM) genes in majority of the cases.

Peripheral T Cell Lymphoma, Non-specified

Peripheral T cell lymphoma (PTCL) is a type of mature T cell neoplasm and present with generalized lymphadenopathy along with organ involvement. Most of the PTCL patients are adult.

Cytology

FNAC smears show discrete medium sized to large cells with pleomorphic nuclei and prominent nucleoli. The nuclei of the cell shows marked irregularity of the membrane with deep notching and convolution. The nuclear chromatin is coarsely clumped with parachromatin clearing.

Immunopheotyping

Positive for CD3, CD7 and CD4/CD8 or co-expression of both.

Hodgkin's Lymphoma

Hodgkin lymphoma was first time described by Thomas Hodgkin in 1832 and further recognized as a specific entity by Samuel Wilks.[74, 75] HL is almost always lymph nodal in origin. Patient of any age group may have HL. There is a bimodal distribution of HL: one peak of incidence in 15–30 years of age another in older age. The patient may have fever, weight loss and other systemic symptoms. The consistency of the lymph node is soft to hard depending on the fibrosis of the node. HL is of B cell in origin.

BOX 28.23 World Health Organization's classification of Hodgkin's lymphoma

Classical Hodgkin Lymphoma
- Nodular sclerosis Hodgkin lymphoma
- Lymphocyte-rich Hodgkin lymphoma
- Mixed cellularity Hodgkin lymphoma
- Lymphocyte-depleted Hodgkin lymphoma

Nodular lymphocyte predominant Hodgkin lymphoma

WHO classification of HL is mentioned in **Box 28.23**.[68]

Classic subtype of HL represents near bout 95% of HL. There is no special clinical significance of recognition of the variants of HL.[76]

Cytomorphology[77,78] (Figs 28.43 to 28.48)

Classical Hodgkin's Lymphoma

Smear of classical HL shows polymorphic population of cells consisting of mature lymphocytes, eosinophils, plasma cells and immunoblasts (**Box 28.24**). Excess eosinophils in the FNAC smear immediately draws attention. The presence of classical RS cells is characteristic of HL. RS cells, are large cell of 20–50 µm diameter. The cells have abundant amphophilic cytoplasm and bi- or polylobed nuclei. The nuclei may truly be bi- or multinuclated. The nuclei are vesicular with coarse clumped chromatin. Each lobe of nuclei show a very large round shaped acidophilic central nucleoli. In a classical RS cell, the two nuclear lobes face with each other containing the prominent nucleoli resulting the mirror image with each other. This is also known as owl-eyed appearance. In case of mononuclear variants of RS cell the cell contains single nucleus with all the above features as described. The RS cell may be very large with large pleomorphic nuclei simulating the cells of anaplastic carcinoma or pleomorphic sarcoma. Many other cells may simulate RS cell (**Box 28.25**). Lymphocyte and histiocyte (L&H) cell or popcorn cell are noted in lymphocyte predominance type of HL. In addition, there may be ill-formed epithelioid granulomas in FNAC smear. It may be difficult to get adequate material in nodular sclerosing variety of HL. The smear of nodular sclerotic variety shows scanty cellularity. The smears may have increased amount of fibroblasts and collagenous material and lacunar type of RS cells. The classical RS cells are also infrequent in this variety of HL. The various other cells may simulate RS cells such as immunoblasts, cells of IM and malignant melanoma. The immunoblasts have irregular nuclear contour, peripheral or marginated basophilic colored nucleoli.

The presence of epithelioid cell granulomas may create diagnostic difficulties with granulomatous inflammation and HL. A careful search for RS cells may be helpful in this aspect. The presence of bi-nucleated large cells simulating RS cell may be a serious diagnostic problem to differentiate ALCL and HL. HL shows a polymorphic population of cells with excess eosinophils.

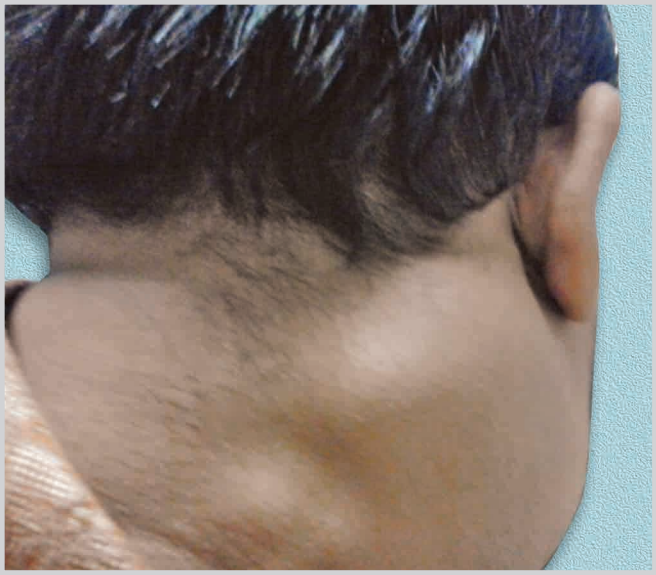


Fig. 28.43: Massive enlargement of neck node in a case of Hodgkin's lymphoma

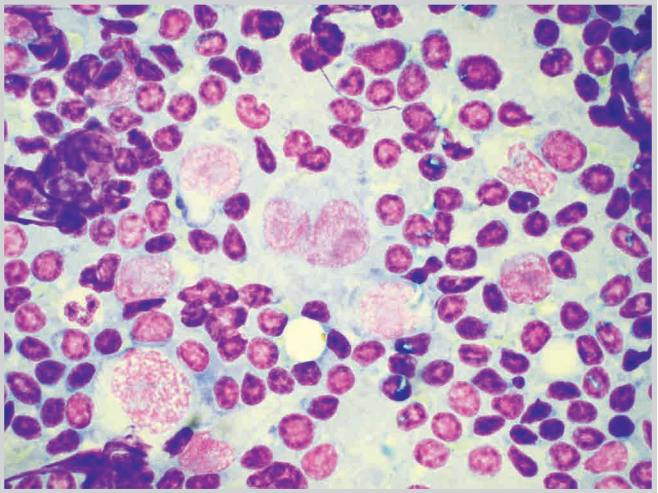


Fig. 28.46: Higher magnification showing classical Reed-Sternberg cell. Note the bi-nucleated cell with moderate amount of cytoplasm and nucleus shows large prominent nucleoli (MGG × OI)

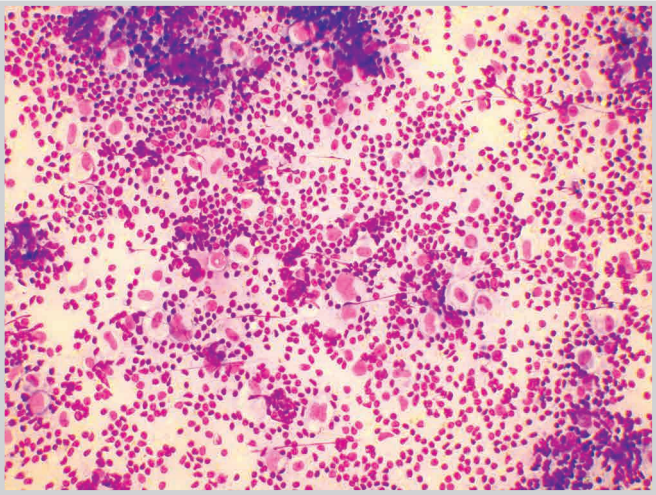


Fig. 28.44: Fine needle aspiration cytology (FNAC) smear of Hodgkin's lymphoma showing polymorphic population of reactive lymphoid cells along with Reed-Sternberg cell and mononuclear Hodgkin cells (MGG × MP)

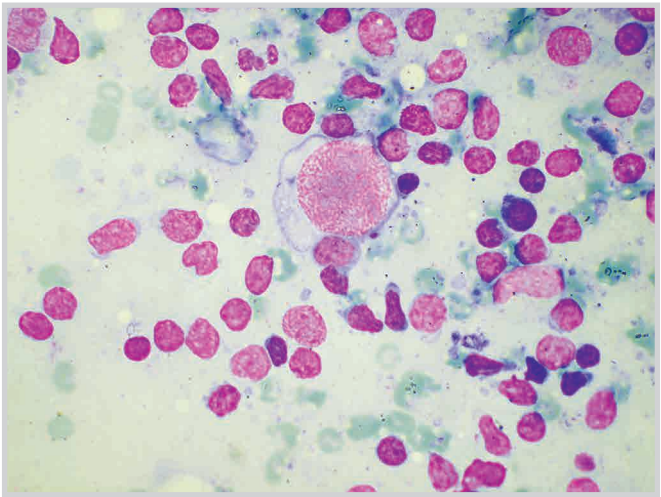


Fig. 28.47A: Higher magnification of large mononuclear Hodgkin cell in Hodgkin's lymphoma (MGG × OI)

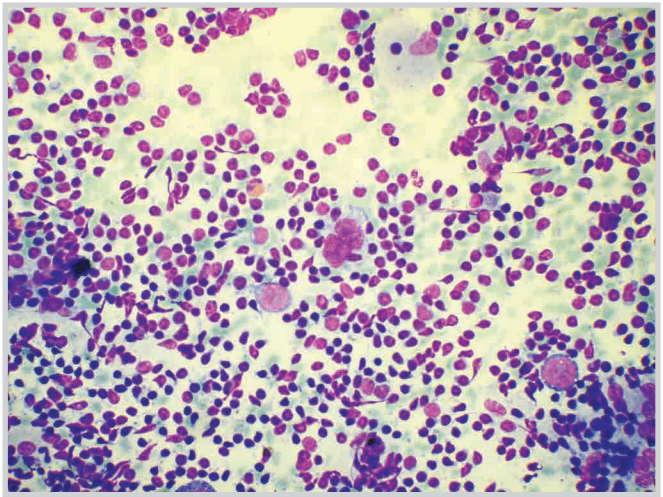


Fig. 28.45: Classical Reed-Sternberg cell along with mixed population of reactive lymphoid cells in Hodgkin's lymphoma (MGG × HP)

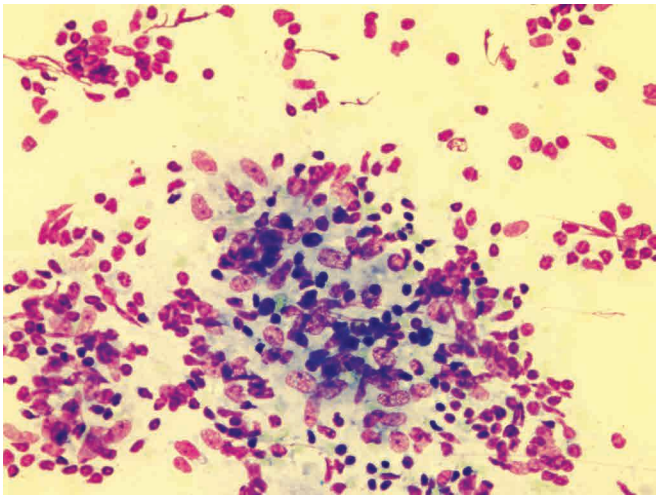


Fig. 28.47B: Hodgkin's lymphoma: Ill-formed epithelioid cell granuloma (MGG × HP)

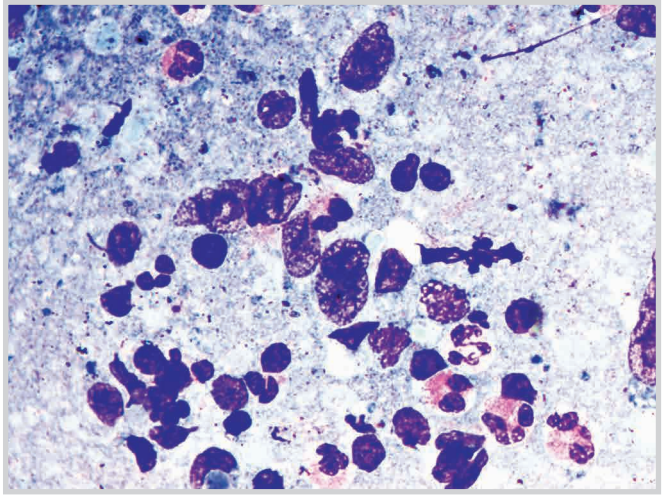


Fig. 28.48: Hodgkin's lymphoma: Many mononuclear Hodgkin cell in lymphocytic depletion type (MGG × OI)

BOX 28.24 Hodgkin's lymphoma

- Polymorphic population
- Mature lymphocytes, eosinophils, plasma cells and immunoblasts
- Classical Reed-Sternberg cells: Large bi-nucleated cell with prominent nucleoli
- Many mononuclear large atypical Hodgkin's cells
- Lymphocyte and histiocyte (L&H) cell/popcorn cell in lymphocyte predominance type
- Ill-formed epithelioid cell granulomas
- Scattered elongated cells in nodular sclerosing variety

BOX 28.25 Reed-Sternberg cells

- RS cell is essential for primary diagnosis of HL

Morphology:

- Large cell
- Abundant cytoplasm
- Bilobed nuclei or double nuclei
- Peripheral coarsely clumped chromatin
- Single macro-nucleoli, basophilic in MGG
- Mirror image nuclei

Variants: Suggestive but not diagnostic of HL

- Mononuclear variants of RS cell
- Multilobated anaplastic type
- Mummified
- Popcorn type

Immunocytochemistry

- CD15+, CD30+, CD45–

Differential diagnosis

- Immunoblasts, infectious mononucleosis, malignant melanoma

TABLE 28.5: Distinguishing immunological markers between Reed-Sternberg cell and lymphocytic and/or histiocytic cells

Immunostain	*Lymphocytic and/or histiocytic cells*	*Reed-Sternberg cell*
CD20	Positive	Negative
CD45	Positive	Negative
CD15	Negative	Positive
CD30	Occasional positive	Positive
EMA	Often positive	Negative

Abbreviation: EMA, epithelial membrane antigen

However, it may not be possible to differentiate these two entities based on cytology alone and detailed immunophenotyping is necessary, particularly in cell block.

Nodular Lymphocytic Predominant HL (NLPHL)

It is a less common type of HL and most of the patients present with localized lymphadenopathy.

Smear of nodular lymphocytic predominant HL (NLPHL) shows abundant small lymphoid cells along with occasional L&H cells. These cells are large with scanty amount of cytoplasm and multilobated extremely folded popcorn-like nucleus with thin nuclear membrane. The nuclear chromatin is vesicular with multiple small nucleoli.

L&H cells are positive for CD20 and CD45, in contrast to classical RS cells that are negative for CD20 and CD45. L&H cells also are negative for CD15 and occasionally positive for CD30 (**Table 28.5**).

At times, it may be difficult to differentiate RLH from NLPHL because dense population of lymphocytes may hide L&H cells. A careful search of L&H cell may be helpful.

Differential Diagnosis

- Granulomatous inflammation
- Anaplastic large cell lymphoma
- Reactive lymphoid hyperplasia
- Nasopharyngeal carcinoma
- T cell-rich B cell lymphoma
- Infectious mononucleosis

APPROACH TO DIAGNOSIS OF LYMPH NODE LESIONS

The **Figures 28.49** and **28.50** show the overall cytological and immunocytochemical approach to diagnosis of lymph node lesions.

The following ancillary investigations are necessary for the exact diagnosis and subtyping of lymphoproliferative disorders:

- Flow cytometric immunophenotyping
- Immunocytochemistry on cell block (supplementary to FCI)
- Proliferative or S-phase cell fraction
- Molecular cytogenetics

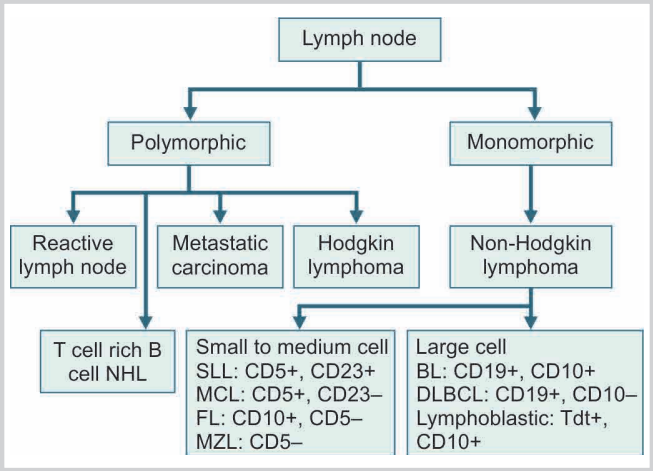


Fig. 28.49: Diagram showing overall approach in the diagnosis of fine needle aspiration cytology (FNAC) of lymph node

BOX 28.26 Basic panel of markers for lymphoma subtyping

Panel for small cell morphology
- Light chain: Kappa and Lambda for B cell clonality
- CD45: Majority of the lymphoid cells
- CD19: B cell
- CD20; B cell
- CD3: T cell
- CD10:
- CD5: T cell, CLL, MCL
- CD23: SLL/CLL

Panel for medium to large cell (additional)
- CD10
- Tdt
- CD15
- CD30

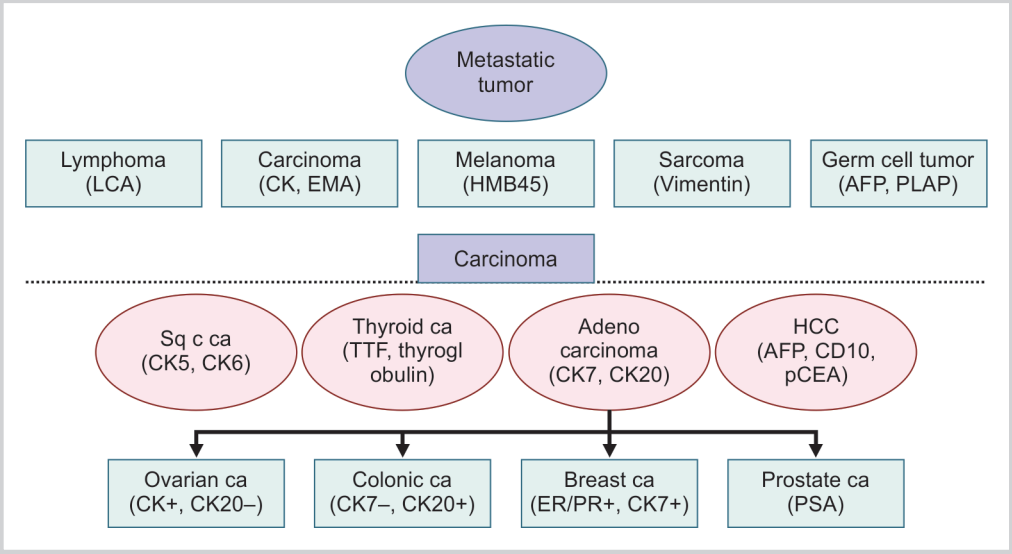


Fig. 28.50: Diagram showing overall approach of immunocytochemistry application in the diagnosis of metastatic tumor in the lymph node aspirate
Abbreviations: Sq c ca, squamous cell carcinoma; ca, carcinoma; HCC, hepatocellular carcinoma

Flow Cytometry

Detailed description and utility of FCI has been already discussed in previous chapter 20. Overall this is now almost mandatory for diagnosis and subtyping of lymphomas. However, its main disadvantage is loss of cytomorphology. FCI may not be helpful in ALCL and HL.

After initial assessment of cytomorphology of the cell, a panel of antibodies should be used for flow cytometric immunophenotyping (**Box 28.26**). In case of NHL with large cell morphology, it is advisable to proceed for immunocytochemistry because of fragile nature of the cell and high false positive result.

Imunocytochemistry

Immunocytochemistry can be done on cell block section or cytospin preparation. The morphology of the cell is well preserved in case of immunocytochemistry. Immunocytochemistry is recommended in large cell NHL, ALCL, and HL. However, immunocytochemistry technique is time consuming and laborious in comparison to flow cytometry (FCM).

Molecular Diagnostic Tests

Molecular diagnostic tests can be performed from FNAC material.[1] The following molecular tests may be necessary
- Fluorescent in situ hybridization (FISH)
- Polymerase chain reaction
- Microarray to see gene expression profile.

Molecular genetics have assisted in the diagnosis of many lymphomas (**Box 28.27**). Translocation of t(14;18) and BCL2 expression are useful in distinguishing RLH from FL. In case of MZL, translocation of t(11;14) is diagnostic. Similarly, translocation of t(8:14) is the diagnostic hall mark of BL. ALCL,

BOX 28.27 Chromosomal alterations in lymphomas

Diagnosis
- MCL: t (11;14)
- MALT lymphoma: t(11:18)
- SLL/CLL: triosomy 12
- FCC lymphoma: t(14:18)
- DLBCL: t (3:14)
- BL: t(8:14)
- ALCL: t(2:5)

Prognosis
- MALT lymphoma the t(1;14) and t(11,18) translocations: bad prognosis
- t(2;5) translocation and over expression of ALK in ALCL: better prognosis
- DLBCL with BCL-10 and CD10 expression: better prognosis

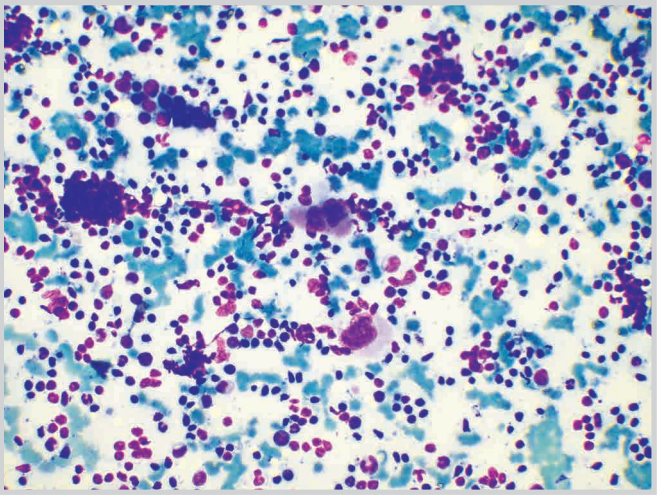


Fig. 28.51: Chronic myeloid leukemia infiltration in the lymph node showing myelocytes, metamyelocytes and megkaryocytes (MGG × MP)

also shows the specific t(2;5) translocation. Molecular genetics also has definite role in assessing prognosis of NHL. It has been noted that t(2;5) translocation and over expression of ALK in ALCL bears a good prognosis. In case of gastric MALT lymphoma the t(1;14) and t(11,18) translocations and over expression of BCL-10 has an adverse prognosis.[79]

It has been shown that a subset of DLBCL with BCL-10 and CD10 expression conveys better prognosis.[80,81]

Leukemic Infiltration[82]

All types of leukemia may involve the lymph nodes. A prior history of leukemia or at least peripheral blood smear should be present at the time of interpretation of the FNAC.

Acute lymphoblastic leukemia: The FNAC smear shows discrete blasts which are often admixed with mature lymphocytes. The blasts are round with scanty deep blue cytoplasm. Nuclei are round with fine chromatin and inconspicuous nucleoli. There is no clinical relevance to separate lymphoblastic lymphoma from a leukemic infiltration in the lymph node.

Acute myeloid leukemia: Infiltration by acute myeloid leukemia shows large number of blasts along with variable number of nucleated RBCs, myelocytes, metamyelocytes and promyelocytes. The presence of Auer rods within the cytoplasm confirms the presence of myeloblasts; however, they may not be noted in every case. In case of promyelocytic leukemia, large numbers of promelocytes are seen in the smear. Cytochemistry or immunophenotyping in the bone marrow aspirate is needed for the primary diagnosis of leukemia.

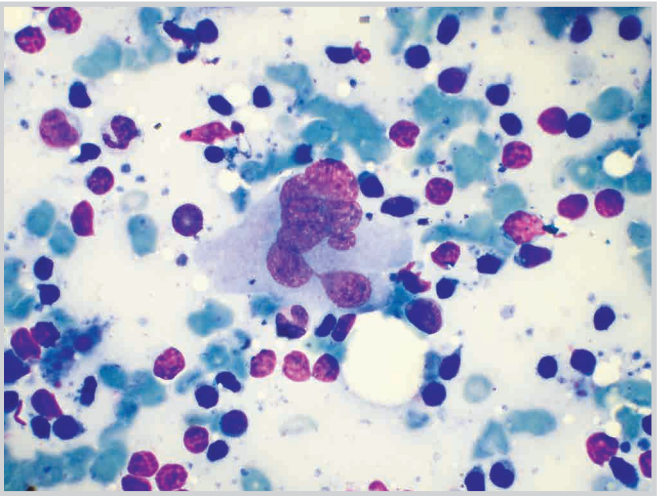


Fig. 28.52: Higher magnification showing megakaryocyte and immature myeloid cells (MGG × HP)

Chronic myeloid leukemia (**Figs 28.51** and **28.52**)*:* In chronic myeloid leukemia, the cytology smear shows variable population of cells of myeloid series along with nucleated RBC, basophil and megakaryocytes. In case of CML with blast crisis, a large number of blasts are seen in the FNAC smear.

There is no clinical relevance to distinguish infiltration by CLL in the lymph node from SLL.

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CHAPTER 29

Mediastinum

Chapter Contents

ANATOMY OF THE MEDIASTINUM AND GENERAL CONSIDERATIONS

The mediastinum is the anatomical space bounded laterally by the pleura of right and left lung, anteriorly by the sternum and posteriorly by the thoracic vertebra, superiorly by thoracic inlet and inferiorly by the diaphragm. The mediastinum is arbitrarily divided into four parts: (1) anterior, (2) posterior, (3) superior, (4) middle mediastinum. The mediastinum contains heart, great vessels and nerves. The topography of mediastinal lesions is important because certain tumors have predilection in particular part of the mediastinum. Thymoma, thymic carcinoids and thymic lymphomas are more frequently seen in superior mediastinum. Certain tumors such as thymoma, lymphoma and germ cell tumors are common in the anterior mediastinum. Lymphomas and metastatic tumors are more frequent in the middle mediastinum. Tumors of neurogenic origin are more frequent in the posterior mediastinum (**Fig. 29.1**).

The mediastinum contains several different types of tumors with variable cytomorphology and often creates diagnostic dilemmas.[1-8] The cytological diagnosis of the mediastinal lesions should always be correlated with a combination of clinical, radiological and biochemical findings.

CLINICAL HISTORY

Majority of the mediastinal lesions is asymptomatic and detected in routine screening or during screening for some other purposes. The common complaints of the patients are cough, chest pain, dyspnea, hemoptysis and superior vena caval syndrome due to the pressure effect on the great vessels. Younger patients are commonly affected by the germ cell tumors and thymoma. Thymoma is often associated with clinical history of the myasthenia gravis. Patients may often give the history of primary malignancy in the lung, breast, kidney, ovary and testis. The patients with generalized lymphadenopathy and or hepatosplenomegaly on physical examination may indicate lymphoma or leukemic involvement in the mediastinal lymph nodes.

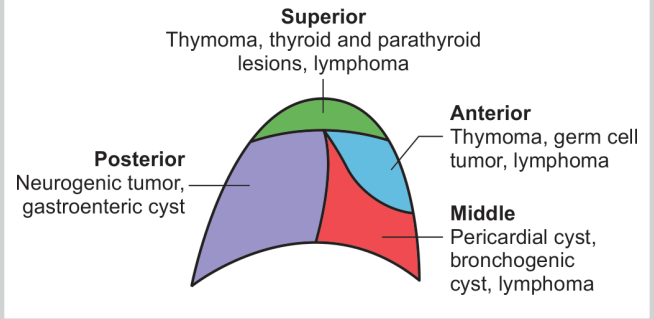

Fig. 29.1: Schematic diagram showing relative preponderance of different tumors in different part of the mediastinum

TECHNIQUES

The mediastinal fine needle aspiration cytology (FNAC) should be done as a team approach of cytologists, radiologists, surgeons and general physicians. The radiologists should localize the lesion and put the needle accordingly. FNAC is done with the help of computed tomography (CT), ultrasonography (USG) or fluoroscopic guidance. CT guided FNAC provides excellent resolution and it is helpful to aspirate from the small lesions. It also helps to avoid the puncture of great vessels. In comparison, USG guided FNAC provides real-time monitoring and it is free of radiation exposure. Transbronchial FNAC of the mediastinal swelling can also be done in case of central lesions around the bronchus. This technique also gives real-time imaging and assessment of the lesion during the time of the procedure. It has added advantage to get material from the small lesions of the mediastinum.

There are no absolute contraindications of mediastinal FNAC. However, it should better be avoided in case of bleeding disorders. FNAC of the mediastinum is usually free of any serious complication. However, hemoptysis, localized bleeding and minor pneumothorax are common complications of FNAC. The pneumothorax can be managed conservatively.

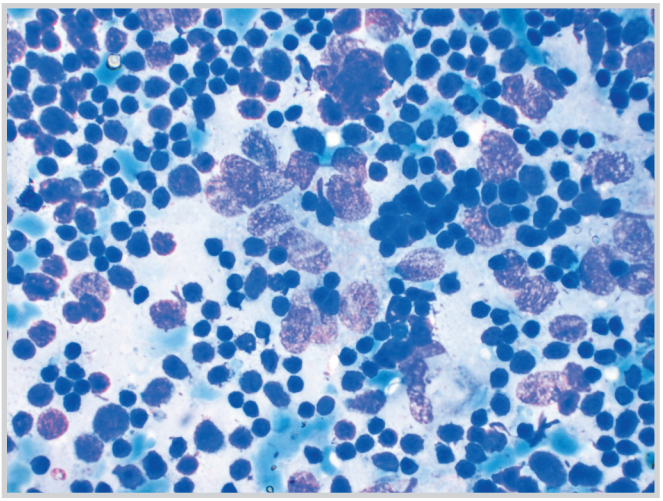

Fig. 29.2: Abundant reactive lymphoid cells along with scattered epithelial cells in thymoma [May-Grünwald-Giemsa (MGG) × high power (HP)]

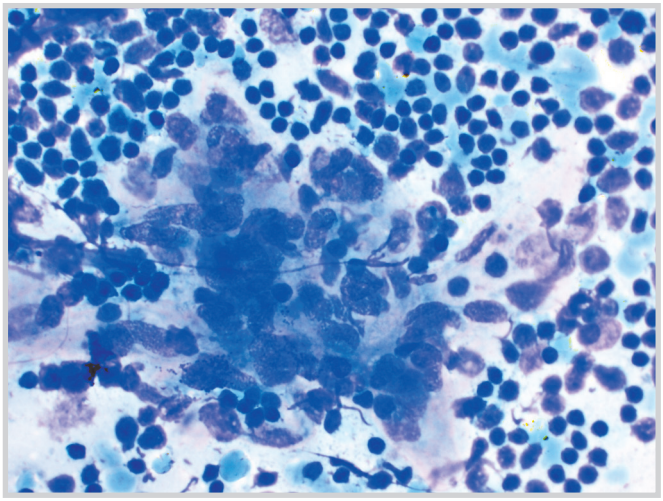

Fig. 29.3: Cluster of oval to elongated epithelial cells with moderate amount of cytoplasm in thymoma (MGG × HP)

LESIONS OF THE MEDIASTINUM

Thymoma[9-11]

Thymoma is the neoplasm that develops from common thymic epithelial stem cell of endodermal origin. This is one of the unique tumors in the mediastinum. It commonly occurs in adults. The thymoma is the most common in the anterior and superior mediastinum. The patients of thymoma usually complain of local pressure symptoms of the mass such as pain, tachycardia, superior vena caval syndrome and respiratory distress. Thymoma is often associated with myasthenia gravis. In addition, thymomas are also associated with large number of autoimmune disorders such as autoimmune hemolytic anemia, autoimmune hepatitis, myositis, myocarditis, dermatomyositis, lupus erythematosus, rheumatoid arthritis, scleroderma, Addison's disease, Graves' disease, etc.

Cytology (Figs 29.2 to 29.4)

According to recent world health organization (WHO) classification thymoma is classified as:[12]

A. Predominant spindle shaped neoplastic epithelial cells and
B. Predominant round or polygonal epithelial cells.

The B-cell component is further subdivided into B1, B2 and B3 depending on the degree of atypia and lymphocytic infiltrate. Now, depending on the admixture of the A and B components the thymoma is classified as A, B or AB. It is generally seen that thymomas with a predominantly spindly epithelial component behaved as benign tumor, whereas thymomas that contain polygonal epithelial cells behave in an aggressive manner.

The term "malignant thymoma" is used in the cases of advanced thymomas that are locally invasive or show metastasis. Thymic epithelial tumors with marked nuclear atypia are also designated as malignant thymoma, irrespective of stage (**Box 29.1**).

Thymic carcinoma shows marked nuclear atypia and features of malignancy.

Fine needle aspiration cytology of thymoma depends on the type of thymoma.[10] Cytology smear shows dual population of multiple clusters of epithelial cells in the background of lymphoid cells. It is easy to diagnosis the cases that contain mixed population of lymphocytes and epithelial cells.

The epithelial cells in thymoma are arranged singly or as large clusters. The clusters are large and irregular in shape and size (**Box 29.2**). Occasional clusters may show cyst-like spaces within them. The clusters of these cells surrounded by arborizing capillary networks are also noted.

The individual epithelial cells are three to four times larger than lymphocytes. The epithelial cells are widely variable in size and shape. They are oval to spindle shaped depending on the type

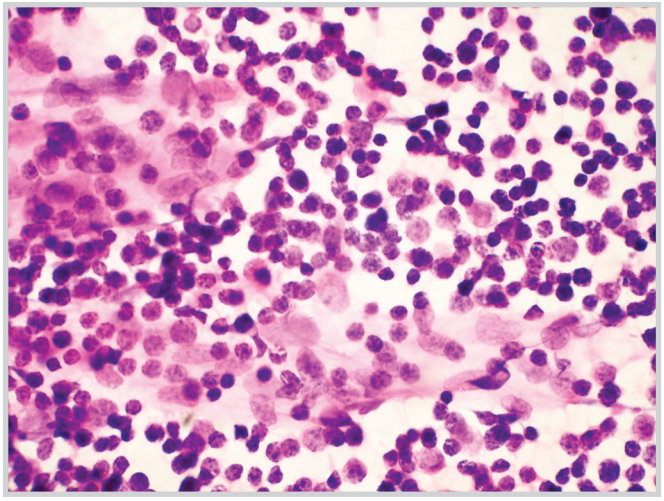

Fig. 29.4: Better morphology of epithelial cells in hematoxylin and eosin stained smear [hematoxylin and eosin (H & E) × HP]

BOX 29.1 Malignant thymoma: any feature

- Locally invasive
- Pleural or pericardial implants
- Distant metastasis
- Marked nuclear atypia

BOX 29.2 Thymoma

- Clusters of oval to polyhedral epithelial cells
  - Moderate cytoplasm with indistinct cell margins
  - Nuclei are vesicular with pale homogenous chromatin
  - Absent nucleoli
- Elongated spindle cells in small fascicles
- Background lymphoid cells
- Immunocytochemistry: Epithelial cells of thymomas are positive for cytokeratin, epithelial membrane antigen (EMA), and carcinoembryonic antigen (CEA).

of thymoma. The cells have moderate cytoplasm with indistinct cell margins. The nuclei are vesicular with pale homogenous chromatin and single prominent nucleoli. The background of the smear shows single naked cells with stripped of cytoplasm.

Abundant monomorphic populations of small, mature lymphocytes along with lymphoglandular bodies and tingible body macrophages are also noted. The nuclear chromatin of lymphocytes is condensed with inconspicuous nucleoli. Occasional large transformed lymphocytes are also seen. Lymphocytes in thymoma are T-cell in origin. Hassall's corpuscle is uncommonly seen in thymomas; however, its presence is characteristic of this lesion.

Spindle cell variant of thymoma shows oval to elongated spindle cells in small clusters. The cells are arranged in small fascicles that are better appreciated in cell block preparation. Variable amount of mature lymphocytes are seen in the background.

Lymphocyte rich thymoma in FNAC shows predominantly lymphocytes and scanty epithelial cells. The presence of large number of lymphoid cells in the thymoma often gives rise to the diagnostic possibility of non-Hodgkin's lymphoma (NHL). The cells in NHL are immature lymphoid cells. Cystic thymoma yields drops of clear or yellowish fluid with sparse cellularity.

Immunocytochemistry: Epithelial cells of thymomas are positive for cytokeratin (CK), epithelial membrane antigen (EMA) and carcinoembryonic antigen (CEA). Thymic tumor cells are strongly positive for AE1-defined acidic CKs and negative for AE3-defined basic CKs. The epithelial cells also show focal positivity for CD20, the B-cell marker.

Differential Diagnosis

- Non-Hodgkin lymphoma
- Hodgkin lymphoma
- Metastatic carcinoma: The cells in metastatic carcinoma show significant nuclear enlargement and pleomorphism. The clinical history of primary malignancy is also an important clue.
- Neurogenic tumors: Spindle cell variant of thymoma may simulate the peripheral nerve sheath tumors. Neural sheath tumors are seen in posterior mediastinum. In addition, the cells of nerve sheath tumor have long slender nuclei with pointed ends along with characteristic nuclear kinking.

Malignant Thymoma[13-15]

Malignant thymoma may be invasive thymoma or thymic carcinomas.

Invasive Thymoma

This tumor is aggressive and shows **c**ytological evidences of malignancy, invasion or metastasis.

Cytology

On FNAC smear, it is almost impossible to detect an invasive thymoma.

Cytology smears show epithelial cells with bland nuclei and background lymphoid cells. Occasionally, the cells may show large nuclei and prominent nucleoli.

Thymic Carcinoma

It is a relatively rare malignancy of thymus and predominantly involves the adults. The unique features of thymic carcinomas are:

- No association with myasthenia gravis
- Rarely associated with autoimmune diseases
- Positive for CD5, CD70 and CD117
- Almost always invasive
- Mostly unresectable tumor.

The epithelial element of thymus undergoes malignant changes. The various morphologic types of thymic carcinomas are

squamous cell carcinoma, anaplastic carcinoma, small cell carcinoma, lymphoepithelial carcinoma, etc.

Cytology

Fine needle aspiration cytology smears of thymic carcinoma usually show abundant epithelial cells with moderate nuclear enlargement and pleomorphism. Nuclei show marked hyperchromasia with prominent nucleoli. Frequent mitotic activities and necrosis may also be seen. Immunocytochemistry shows positivity for CD5, CD70 and CD117.

Differential Diagnosis

- Metastatic carcinoma: It is impossible to differentiate primary thymic carcinoma from metastatic carcinoma on the basis of cytomorphology. Clinical history of primary carcinoma is helpful in this aspect.

Thymic Carcinoid

Thymic carcinoid develops from the primitive cells with neuroendocrine differentiation. These are rare tumors and represent only 2–5% of all thymic tumors.[12] Thymic carcinoids are classified as:

- Well-differentiated neuroendocrine carcinoma (NEC):
  - Typical carcinoids
  - Atypical carcinoids
- Poorly differentiated NEC.

The patient often presents with various endocrine manifestations.

Cytology (Fig. 29.5)

Cytologic appearance of thymic carcinoid is similar as seen in other sites. The smear shows round to oval discrete cells with occasional rosettes-like arrangement (**Box 29.3**). The individual cells show moderate amount of reddish granular cytoplasm. Nuclei are round and regular with typical salt and pepper chromatin with inconspicuous to absent nucleoli. Variable amount of spindle cells are also noted. Occasional pseudonuclear cytoplasmic inclusions may also be seen.

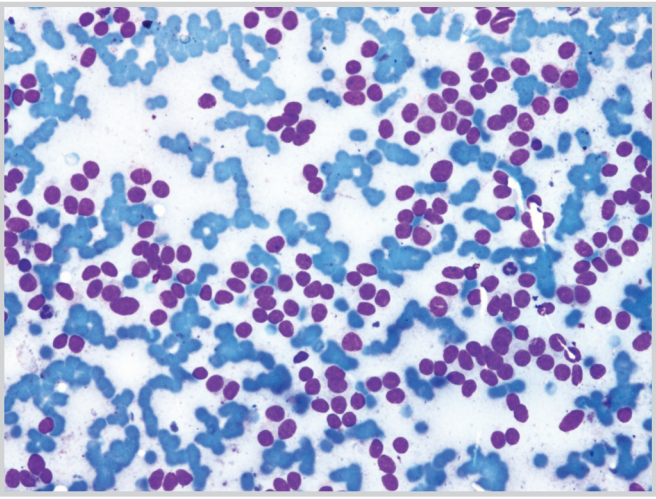

Fig. 29.5: Monomorphic round to oval discrete cells in thymic carcinoid [MGG × medium power (MP)]

BOX 29.3 Thymic carcinoid

- Dissociated cells
- Round cells with moderate amount of reddish granular cytoplasm
- Round nuclei, minimal pleomorphism
- Salt and pepper chromatin and inconspicuous nucleoli
- Spindle cells

Atypical Carcinoid

Cytomorphology of the atypical carcinoid shows abundant dissociated round cells with moderate nuclear enlargement and pleomorphism. Occasional cells may show nuclear molding. In addition, the smears show high mitotic activity and necrosis.

Neuroendocrine carcinoma of thymus is positive for the neuroendocrine markers such as chromogranin, synaptophysin. The cells are also positive for CK.

Differential Diagnosis

- Small cell carcinoma: Rosette like arrangement, cells with moderate amount of cytoplasm and salt and pepper like appearance of nuclear chromatin indicate the diagnosis of carcinoid.
- Non-Hodgkin lymphoma: Immature lymphoid cells of NHL may simulate carcinoid. Individual cell morphology and immunocytochemistry are helpful in diagnosis.

Germ Cell Tumors[16,17]

Mediastinum is the most frequent site of the origin of the extragonadal germ cell tumors. The germ cell tumor is commonly seen in the anterior mediastinum. It is worthwhile to mention that metastatic germ cell tumor is more common than the primary germ cell tumor. Therefore, one should be careful to search the presence of the primary tumor in the gonads first in case of mediastinal germ cell tumors.

Mature Cystic Teratoma

Mature cystic teratoma is the commonest germ cell tumors in the mediastinum. It represents 7–9% of all mediastinal germ cell tumors. It predominantly occurs in young adults and the mean age of the adult is 28 years. Teratoma may be solid or cystic. The patients are usually asymptomatic. The chief complaints of the patients are chest pain, fever or cough.

Cytology

Fine needle aspiration cytology smear of mature cystic teratoma usually yields pultaceous cheesy material. The smear shows aneucleated squames and mature squamous cells (**Box 29.4**).

BOX 29.4 **Mature cystic teratoma**

- Mature squamous cells
- Anucleated squames
- Keratin flakes, skin adnexal cells
- Glandular cells
- Neural elements

There may be the other elements of skin, neural structure, respiratory lining cells, glandular elements, etc. The smears also show multinucleated giant cells and epithelioid cell granulomas.

Differential Diagnosis

Squamous cell carcinoma: The presence of mature squamous cells and anucleated squames may often mislead to the false diagnosis of metastatic squamous cell carcinoma.

Immature Teratoma

It is a rare tumor in the mediastinum. The presence of immature neuroectodermal elements such as rosettes and tubules indicate the presence of immature teratoma.

Seminomas

Seminomas are rare germ cell tumors in the mediastinum. They represent only 8% of all extranodal germ cell tumors. Seminoma of the mediastinum is commonly seen in young male and most of the cases occur in the third and fourth decade of life. Seminoma commonly occurs in the anterior mediastinum.

Cytology (Figs 29.6 and 29.7)

Fine needle aspiration cytology smear of the seminoma shows abundant dispersed round cells. Background of the smear shows typical tigroid or stripped appearance (**Box 29.5**). Necrosis and cells with naked nuclei are also noted. The individual tumor cells have abundant vacuolated cytoplasm containing glycogen. The nuclei are large round and moderately pleomorphic. The nuclear chromatin is fine with single to multiple prominent nucleoli. These cells are admixed with many lymphocytes and plasma cell. This characteristic appearance of germinoma is due to rupture of the cytoplasmic content into the background. Mitosis and apoptosis are common. Occasional scattered large "syncytiotrophoblast-like" cells are seen. The smears also show ill-formed epithelioid granulomas.

Immunohistochemistry

Majority of the mediastinal seminomas are positive for placental alkaline phosphatase (PLAP). Large numbers of cases are also positive for vimentin and CD117. Focal weak positivity for pancytokeratin is also seen.

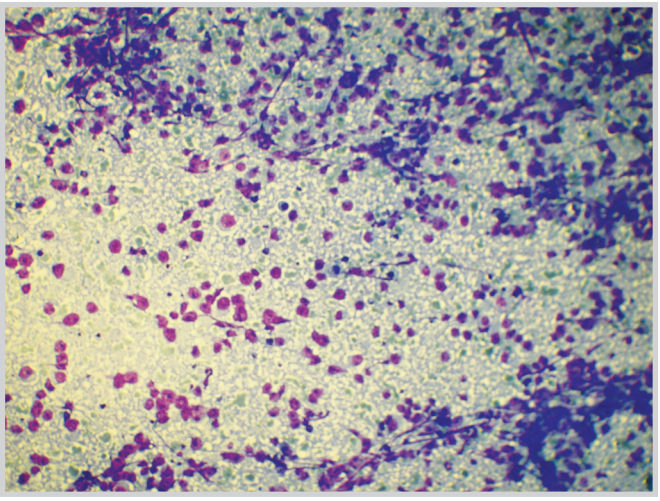

Fig. 29.6: Fine needle aspiration cytology (FNAC) smear of a germinoma in mediastinum showing scattered round cells in a tigroid background (MGG × MP)

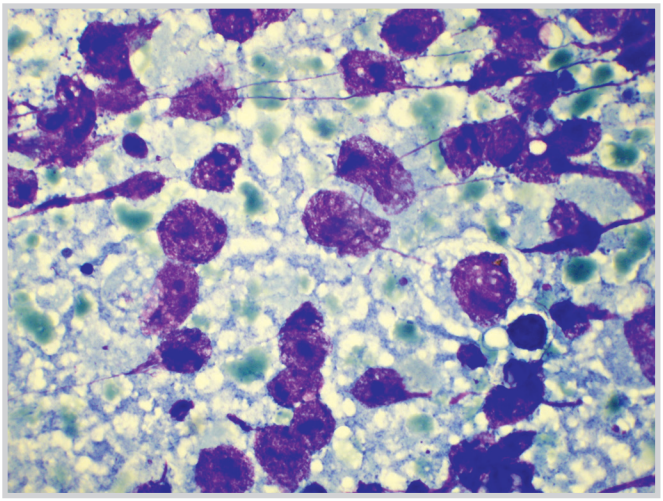

Fig. 29.7: Higher magnification showing individual cell morphology. The cells are round, moderately pleomorphic with fine chromatin and prominent nucleoli (MGG × HP)

BOX 29.5 **Seminomas**

- Discrete cells
- Tigroid background
- Round cells
- Abundant vacuolated cytoplasm
- Moderately pleomorphic nuclei
- Fine chromatin
- Single to multiple nucleoli
- High mitotic activity
- Lymphocytes
- Ill-formed epithelioid cell granulomas

Differential Diagnosis

- Non-Hodgkin's lymphoma
- Poorly differentiated carcinoma
- Other germ cell tumor such as embryonal carcinoma (EC).

Embryonal Carcinoma

Embryonal carcinoma represents about 12% of all germ cell tumors of the mediastinum. It commonly occurs in the young males and the mean age of the tumor is 27 years. Cytology smear of EC is similar to that of the gonad.

Cytology (Fig. 29.8)

Fine needle aspiration cytology smears show multiple small cohesive clusters and discrete malignant cells (**Box 29.6**). The individual cells have scanty to moderate amount of vacuolated cytoplasm with ill-defined cell border. The nuclei of the cells are enlarged and moderately pleomorphic. The nuclei show vesicular chromatin with single to multiple large nucleoli.

Immunocytochemistry

The majority of ECs are positive for CD30.The cells are also positive for CK.

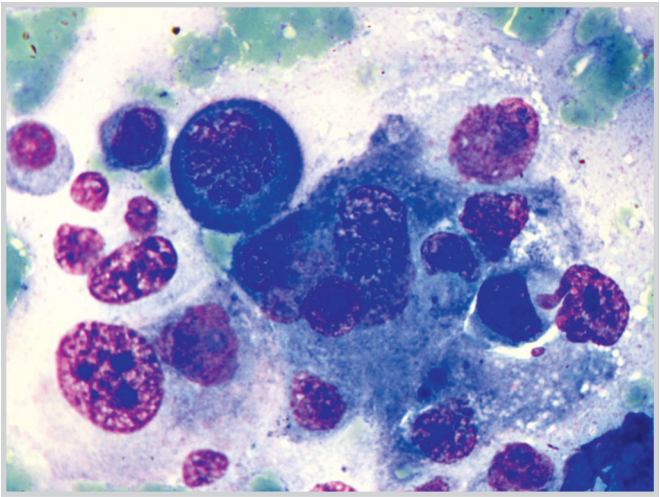

Fig. 29.8: Mediastinal embryonal carcinoma showing markedly pleomorphic cells with multiple prominent nucleoli [MGG × oil immersion (OI)]

BOX 29.6 **Embryonal carcinoma**

- Cohesive clusters of cells
- Large cells with granular to vacuolated cytoplasm
- Moderate to marked pleomorphic nuclei
- Single to multiple nucleoli
- Macronucleoli

Diagnostic Difficulty

- Seminoma
- Poorly differentiated carcinoma.

Endodermal Sinus Tumor

This is a rare tumor in the mediastinum and occurs predominantly in infant and young child. Cytology smear shows small clusters, papillae and discrete malignant cells. The cells are round with vacuolated cytoplasm and mild to moderately pleomorphic nuclei. Nucleoli are relatively small in size. Occasional intracytoplasmic and extracytoplasmic small round pink globules are also seen. Schiller Duval bodies are rarely present in endodermal sinus tumor (EST).

Immunocytochemistry

The cells show low molecular weight CK. The cells are also positive for alpha fetoprotein.

Lymphomas[18-20]

Non-Hodgkin Lymphoma

Non-Hodgkin lymphoma in the mediastinum develops from the mediastinal lymph node or from the thymic lymphocytes. Precursor T-lymphoblastic lymphoma (T-cell) and diffuse sclerosing large B-cell lymphomas are the common types of mediastinal lymphoma.

Diffuse Large Cell Non-Hodgkin Lymphoma

Primary mediastinal large B-cell lymphoma (PMLBCL) is type of diffuse large B-cell NHL that undergoes sclerosis. Primary B-cell lymphomas represent for 2–3% of all NHLs. Unlike diffuse large B-cell lymphocyte (DLBCL), this tumor commonly occurs in young adults. Due to marked sclerosis of the lymph node, the cellular yield may be poor. PMLBCL is seen in the anterosuperior mediastinum. This lymphoma develops from the thymic lymphoid cells and secondarily involves the adjacent lymph nodes of the thymus. Superficial lymphadenopathy and hepatosplenomegaly are not seen in PMLBCL.

Cytology (Figs 29.9 and 29.10)

Fine needle aspiration cytology smear shows dispersed population of large lymphoid cells in the background of lymphoglandular bodies (**Box 29.7**). Occasional small clusters of cells are also seen. The size of the lymphoid cell is three times more than the mature lymphocyte. The cells are round with variable amount of cytoplasm. The nuclei are round, moderately pleomorphic having irregular margin and convolution. Nuclear chromatin is fine with one or more prominent nucleoli located in the peripheral membrane of the nuclei. The tumor shows increased mitotic activity. Occasional spindle cells may be seen. In addition, there may be large cells with abundant cytoplasm and moderately pleomorphic nuclei. These cells may resemble Reed-Sternberg (RS) cells of Hodgkin lymphoma.[3]

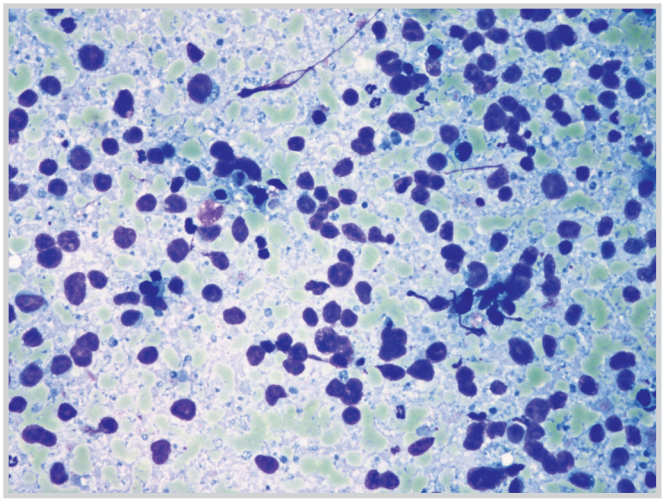

Fig. 29.9: Discrete large round cells in diffuse large B-cell lymphocyte (DLBCL) (MGG × MP)

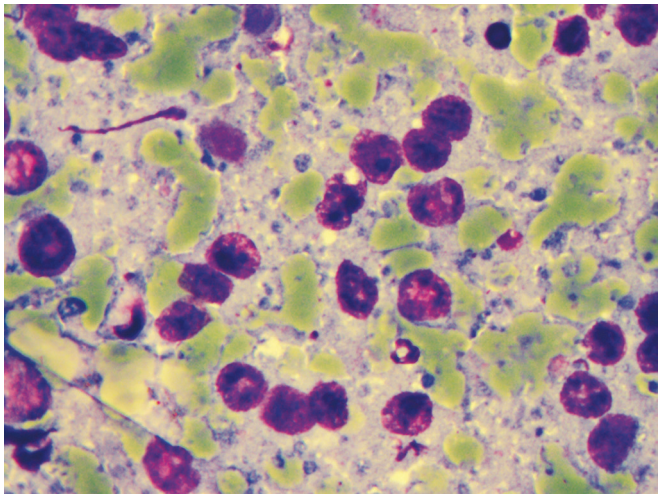

Fig. 29.10: Higher magnification showing enlarged cells, fine chromatin and large prominent nucleoli (MGG × OI)

BOX 29.7 Large cell non-Hodgkin lymphoma

- Discrete large cells, three times larger than lymphocytes
- Variable amount of cytoplasm, minimal to moderate in amount
- Round to convoluted nuclear margin
- Vesicular chromatin, prominent nucleoli
- Lymphoglandular bodies

Immunocytochemistry

Primary mediastinal large B-cell lymphoma expresses B-cell markers such as CD19, CD20 and CD22. Focal weak positivity of CD30 is also demonstrated.

Diagnostic Difficulty

- Seminoma
- Hodgkin's lymphoma
- Spindle cell tumor: Due to sclerosis in this lymphoma, the lymphoid cells often show spindle shaped distortion and may give rise to the mistaken diagnosis of soft tissue sarcomas, spindle cell variant of thymoma and malignant melanoma.
- Metastatic carcinoma: Occasional small clusters of the lymphoid cells may give rise to the wrong diagnosis of poorly differentiated carcinoma.

Lymphoblastic Lymphoma

Precursor T-lymphoblastic lymphoma develops from T-cell. This is common in children and young adults. It is a high-grade aggressive lymphoma that typically involves the thymus and mediastinal lymph nodes.

Cytology (Fig. 29.11)

Precursor T-lymphoblastic lymphoma shows dispersed monotonous population of small to medium sized lymphoid cells (**Box 29.8**). The cells are 1.5–2 times larger than the mature lymphocytes. The individual cells have a scanty thin rim of deep blue cytoplasm with round relatively monomorphic nuclei. The nuclear margin may be irregular. Chromatin is fine reticulated with inconspicuous nucleoli. Increased mitotic figures and many tingible body macrophages are seen in the background.

Immunocytochemistry

The cells are positive for terminal deoxynucleotidyl transferase (TdT) along with T-cell markers such as CD3 and CD5.

Differential Diagnosis

Various small round blue cell tumors such as neuroblastoma, rhabdomyosarcoma, etc. comes in the differential diagnosis of precursor T-lymphoblastic lymphoma.

Anaplastic Large Cell Lymphomas

Anaplastic large cell lymphoma (ALCL) is uncommon in the mediastinum. It usually occurs in children and young adults. FNAC of ALCL has already been described in lymphoma section. The cytology smear shows typical "hallmark" cells characterized by large cells with abundant cytoplasm and kidney shaped or embryo shaped nuclei. Flow cytometry (FCM) is needed to confirm the diagnosis of ALCL. The tumor cells are positive for T-cell markers such as CD3, CD45RO. The cells are also positive for EMA, CD30 and anaplastic lymphoma kinase (ALK).

Hodgkin Lymphoma

Among the different types of Hodgkin lymphoma, Nodular sclerosing Hodgkin lymphoma is the most common primary

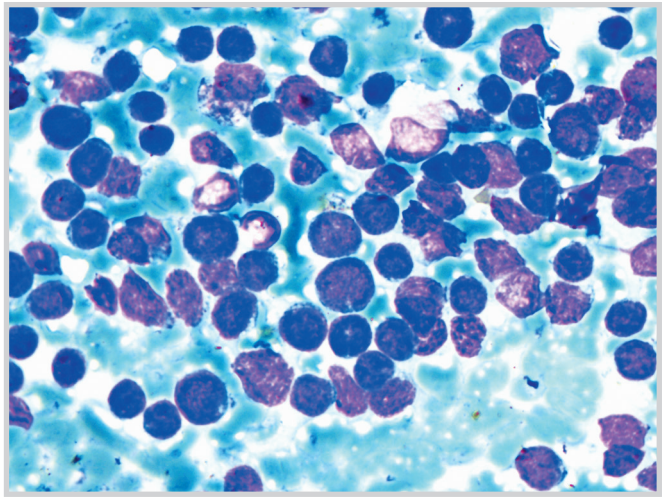

Fig. 29.11: Lymphoblastic lymphoma showing cells with scanty thin rim of deep blue cytoplasm and round relatively monomorphic nuclei with inconspicuous nucleoli (MGG × HP)

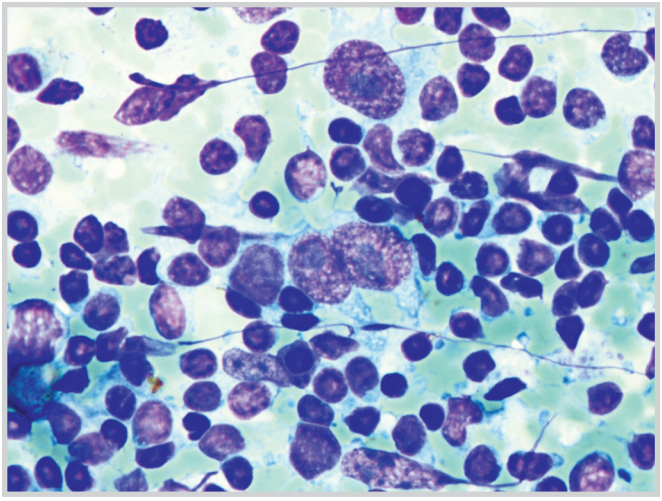

Fig. 29.12: Polymorphic population of cells along with classical Reed-Sternberg cell in Hodgkin lymphoma (MGG × HP)

BOX 29.8 Lymphoblastic lymphoma

- Medium sized cell, twice the size of mature lymphocyte
- Scanty rim of cytoplasm
- Irregular nuclear margin
- Fine reticular chromatin, inconspicuous nucleoli

BOX 29.9 Hodgkin's lymphoma

- Polymorphic population of lymphoid cells
- Classical Reed-Sternberg cell
- Mononuclear Hodgkin's cells

lymphoma in the mediastinum. It affects young adult, particularly young female.

Cytology (Fig. 29.12)

Fine needle aspiration cytology of Hodgkin lymphoma shows similar cytological features as that noted in other superficial lymph nodes. The yield of the cell may be poor in these cases due to sclerosis. The smear shows a polymorphic population of reactive lymphoid cells consisting of mature lymphocytes, follicular center cells, eosinophils, plasma cells and characteristic typical RS cells (**Box 29.9**).

Neurogenic Tumor[21,22]

The neurogenic tumors are commonly located in the posterior mediastinum. Neuroblastoma is the commonest tumor of neurogenic origin in children. In adult, the peripheral nerve sheath tumors are common.

Neuroblastoma

This is a small blue round cell tumor. The salient cytological features of neuroblastomas are described in **Box 29.10**.

Cytology (Fig. 29.13)

Neuroblastoma shows monomorphic small round cells arranged in loose cluster and discretely. Pseudorosettes are also seen. The cells show scanty deep blue cytoplasm with hyperchromatic round monomorphic nuclei having small nucleoli. Neural filamentous material may be seen in the background.

Ganglioneuroblastoma

The cells with ganglionic differentiation are seen in ganglioneuroblastoma. The ganglion cells are polygonal shape with abundant cytoplasm having centrally placed nuclei. The nuclear chromatin is vesicular with prominent nucleoli.

Peripheral Nerve Sheath Tumor

Neurofibroma and Schwannoma

Schwannoma is the most common neurogenic tumor in the posterior mediastinum. Neurofibromas are also seen in the posterior mediastinum, mostly in association with von Recklinghausen's disease.

BOX 29.10 Neuroblastoma

- Dissociated and small cluster
- Pseudo-rosettes
- Small round cell
- Scanty cytoplasm
- Round monomorphic nuclei
- Hyperchromatic
- Small nucleoli
- Neurofibrillary material

BOX 29.11 Neurofibroma and schwannoma

- Small fascicles of cells
- Oval to spindle shaped cells
- Scanty cytoplasm
- Long and slender nuclei
- Thin pointed ends
- Nuclear waving and kinking

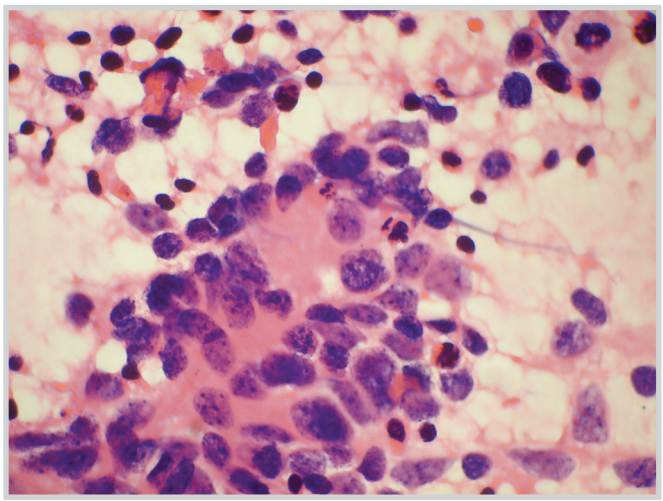

Fig. 29.13: Mediastinal neuroblastoma showing rosette formation (H & E × OI)

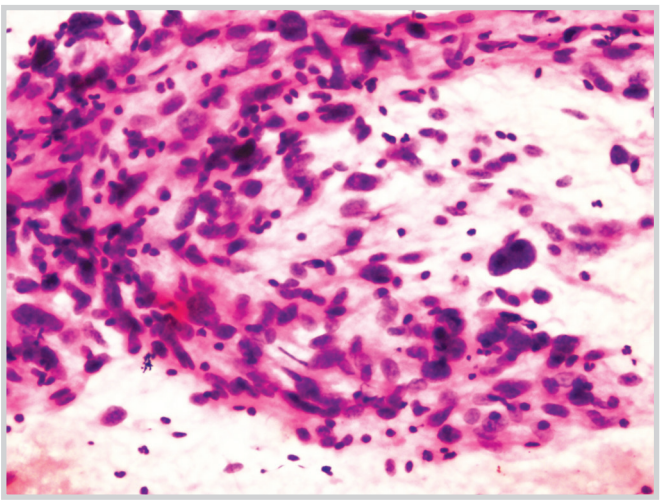

Fig. 29.14: Malignant peripheral nerve sheath tumor of posterior mediastinum showing loose cluster and discrete oval to elongated spindle cells with moderate nuclear pleomorphism (H & E × MP)

Cytology

Fine needle aspiration cytology smear of the peripheral nerve sheath tumor shows small fascicles and dissociated cells. The cells are long slender with elongated wavy nuclei having thin pointed ends (**Box 29.11**). Characteristic nuclear kinking is also seen. Nuclear chromatin is fine with prominent nucleoli. The background of the smear show eosinophilic fibrillar material.

Malignant Peripheral Nerve Sheath Tumors (Fig. 29.14)

The cytomorphology of malignant peripheral nerve sheath tumor (MPNST) has been described in soft tissue tumor section. FNAC smear shows oval to spindle-shaped cells in small clusters. The cells show whorling appearance. The tumor cells are spindle-shaped with elongated moderate to marked pleomorphic nuclei. Mitotic activity is high. Background necrosis is frequently seen.

Diagnostic Difficulties

- Spindle cell type of thymoma
- Large cell sclerosing lymphoma
- Other soft tissue tumors
- Malignant melanoma.

Paraganglioma

Paraganglioma arises from the sympathetic chain of the posterior mediastinum. The detailed cytomorphology has been described in head neck section.

Metastatic Carcinoma

Different types of metastatic tumors may involve the mediastinal lymph nodes. The commonest primary sites of the malignancies are lung, breast, head neck region, gastrointestinal tract and kidney. Clinical history along with detailed cytomorphology is helpful in diagnosis and subtyping of malignancies. In certain situation, detailed immunocytochemistry on cell block section is helpful.

APPROACH TO THE DIAGNOSIS OF MEDIASTINAL TUMORS

As mentioned before, the following data is helpful in the diagnosis of mediastinal lesions:

- Relevant clinical history
- Radiological localization
- Cytomorphology data base
- Ancillary investigations.

TABLE 29.1: Immunocytochemistry of different mediastinal tumors

Lesions	*Immunocytochemistry*				
	CK	*LCA*	*Chromogranin*	*PLAP*	*NB45*
Thymoma	+	–	–	–	
NHL	–	+	–	–	
Seminoma	+ (occasional)	–	–	+	
Neuroblastoma	–	–	–	–	+
Small cell carcinoma	–	–	+	–	–

Abbreviations: CK, cytokeratin; PLAP, placental leukocyte alkaline phosphatase; LCA, leukocyte common antigen; NB, neuroblastoma protein; NHL, non-Hodgkin's lymphoma

Relevant clinical history and radiological localization help to make a short list of diagnostic possibilities. In addition, the basic cytomorphology also helps in the provisional diagnosis of most of the lesions such as:[23]

- Round cell morphology:
  - Cohesive cells: Thymoma, metastatic carcinoma
  - Discohesive cells: NHL, seminoma, Hodgkin lymphoma.
- Spindle cell morphology: Spindle cell thymoma, spindle cell melanoma, neurofibroma or schwannoma, MPNST
- Pleomorphic cells: Metastatic carcinoma, ALCL, EC

Once provisional diagnosis is made, then further ancillary tests may be done to confirm the diagnosis. The common ancillary investigations are:

1. Special stains: Ziehl–Neelsen (Z-N) stain, mucicarmine, etc.
2. Culture: Bacterial and fungal culture
3. Flow cytometry
4. Immunocytochemistry (**Table 29.1**)
5. Others: Polymeric chain reaction (PCR), biochemical markers, etc.

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CHAPTER 30

Liver and Spleen

Chapter Contents

LIVER

Fine needle aspiration cytology (FNAC) of the liver is mainly useful for the diagnosis of neoplastic lesion particularly to distinguish a primary liver tumor from a metastatic one and also to identify the type of metastatic or primary malignancy. FNAC is done under the guidance of ultrasonography (USG), computed tomography (CT) scan or via endoscopy. The diagnostic accuracy of liver FNAC is near about 100%. The sensitivity and specificity of FNAC of liver in the diagnosis of malignancy are about 95% and 100%, respectively. FNAC is of little help in the diffuse parenchymal lesions of the liver such as cirrhosis, hepatitis etc. except in certain conditions such as amyloidosis and hemochromatosis. Occasionally, metastatic malignancy may be diffusely infiltrative such as breast carcinoma and lymphoma. FNAC may be helpful in such conditions [1-3].

USG guidance FNAC of liver can be done from multiple sites in a single setting and the adequate material can be obtained from a lesion as small as 1 cm diameter. A space occupying lesion in the liver may be hepatocellular carcinoma (HCC), metastatic carcinoma, hydatid cyst, amoebic or pyogenic abscess. FNAC diagnosis in these lesions guides the surgeons or physician to manage the case accordingly.

There is almost no complication of the FNAC of the liver lesion. However, rarely focal hemorrhage, biliary peritonitis and anaphylactic shock (in hydatid cyst) may occur. Bleeding diathesis is the contraindication of liver FNAC and therefore prothrombin time or prothrombin index should be done before this procedure. The diameter of the needle preferably should be small and 22–25 gauze needle reduces the risk of any complication. It is better to avoid FNAC from a hydatid cyst as there is a chance of spillage of cyst fluid and development of allergic reaction.

Normal Cells

Normal cellular contents of FNAC of the liver are:

Hepatocytes (**Figs 30.1A** and **B**): Hepatocytes are present in small clusters or discretely. The cells are large polygonal with abundant cytoplasm containing golden brown bile pigment. The nuclei are centrally placed round monomorphic with fine granular chromatin and prominent nucleoli (**Box 30.1**). Pseudoinclusions are also noted. Mild degree of nuclear pleomorphism may be seen in the benign hepatocyte.

Bile ductules (**Fig. 30.2**): The bile ductular cells are present usually in small cohesive clusters. The cells are well spaced and give a honeycomb-like appearance (**Box 30.2**). The individual cells are small with scanty cytoplasm and small round monomorphic nuclei.

Kupffer cells: The Kupffer cells are usually single cells and they present in between the hepatocytes. These are cells of histiocytic

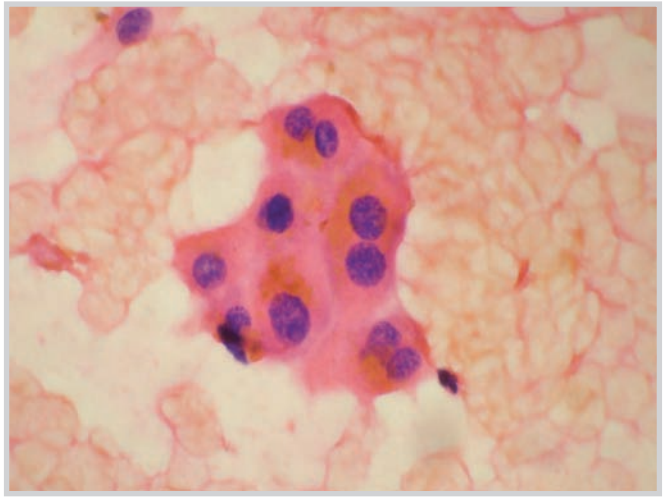

Fig. 30.1A: Benign hepatocytes in cluster. Note the polygonal cells with abundant cytoplasm and centrally placed monomorphic round nuclei. Cytoplasm of the cell contains golden brown bile pigment [hematoxylin and eosin (H & E) stain × high power (HP)]

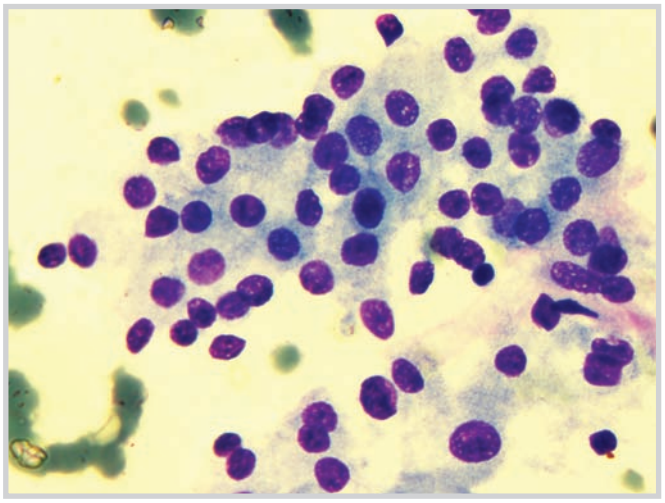

Fig. 30.1B: Benign hepatocytes in cluster. Polygonal cells with abundant cytoplasm having monomorphic nuclei [May-Grünwald-Giemsa (MGG) stain × high power(HP)]

BOX 30.1 Hepatocytes

- Polygonal cells
- Abundant cytoplasm
- Central round nuclei
- Prominent nucleoli
- Nuclear pesudoinclusion
- Bile pigments in cytoplasm

in origin. The Kupffer cells have scanty cytoplasm, bean-shaped nuclei with inconspicuous nucleoli. Phagocytosed materials are often noted within the Kupffer cell.

Endothelial cells: These cells surround the cluster of hepatocytes. Individual cells have spindle-shaped elongated nuclei with prominent nucleoli and scanty delicate cytoplasm.

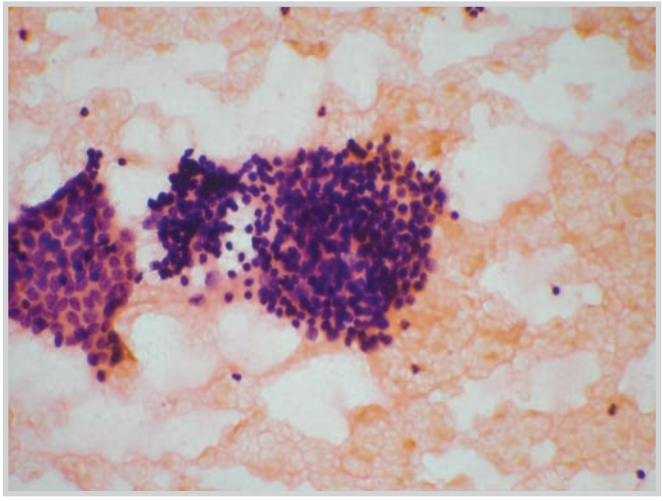

Fig. 30.2: Bile ductular cells in small clusters. The cells are round monomorphic having scanty cytoplasm [hematoxylin and eosin (H & E) stain × medium power(MP)]

BOX 30.2 Bile ductular cells

- Small clusters or honeycomb sheet
- Small round cells
- Scanty cytoplasm

TABLE 30.1: Common space occupying lesions in liver

Common space occupying lesions in liver	
Non-neoplastic • Hepatic abscess • Hydatid cyst • Granulomas • Focal nodular hyperplasia	Neoplastic Benign • Liver adenoma • Cavernous Hemangioma Malignant Primary • Hepatocellular carcinoma • Hepatoblastoma • Cholangiocarcinoma Secondary • Metastatic carcinoma • Metastatic sarcoma

LIVER LESIONS

The common space occupying lesions in the liver encountered by FNAC are listed in **Table 30.1**.

Non-neoplastic Lesions

Cysts

Congenital Cysts

These are usually unilocular. The wall of the cyst is lined by cuboidal to columnar epithelium. FNAC of the congenital cyst yields clear fluid and the smear is usually hypocellular that

shows predominantly macrophages and cuboidal to columnar epithelial cells.

The ciliated foregut cyst is also solitary and remains beneath the falciform ligament. These cysts are lined by ciliated columnar epithelium. FNAC smears of the cyst show mucus cells and ciliated columnar cells. At times the cilia may be lost and the cells may be mistaken as malignant cells.

Hydatid Cysts

Hydatid cyst is caused by *Echinococcus granulosus*, a tape worm. Infection of this parasite invades multiple organs in the human body. Liver is the most common site of hydatid cyst. The cyst in the liver is usually solitary and on imaging the cyst is covered by a membrane and filled with fluid. The membrane is acellular. From the germinal layer many multiple daughter cysts may grow. The daughter cysts contain many scolices with hooklets.

Cytology (Fig. 30.3A)

FNAC from the hydatid cyst is not recommended because of the risk of anaphylactic shock. However it may be done accidentally from the hydatid cyst. FNAC yields clear fluid. The smear shows polymorphs, laminated membrane-like structure, occasional multinucleated giant cells and many scolices with hooklets (**Box 30.3**). The presence of hooklets is diagnostic of hydatid cyst.

Pyogenic Abscess (Fig. 30.3B)

This is usually caused by bacterial infection due to sepsis or ascending cholangitis. The common organisms are streptococci and staphylococci. Cytology smear shows numerous polymorphs, necrotic debris and degenerated hepatocytes. The hepatocytes in the wall of the cyst may show significant reactive atypia (**Box 30.4**).

Amoebic Abscess

This is due to infection of amoeba. FNAC smear shows both polymorphs and lymphocytes along with the amoebic trophozoites. *Entamoeba histolytica* have small vesicular nuclei and vacuolated cytoplasm with phagocytosed red blood cells (RBC).

Benign Lesion

World Health Organization (WHO) has classified the neoplastic lesions of liver as [4]

Epithelial Tumors

Benign

- Focal nodular hyperplasia
- Hepatocellular adenoma (liver cell adenoma)
- Intrahepatic bile duct adenoma
- Intrahepatic bile duct cystadenoma
- Biliary papillomatosis

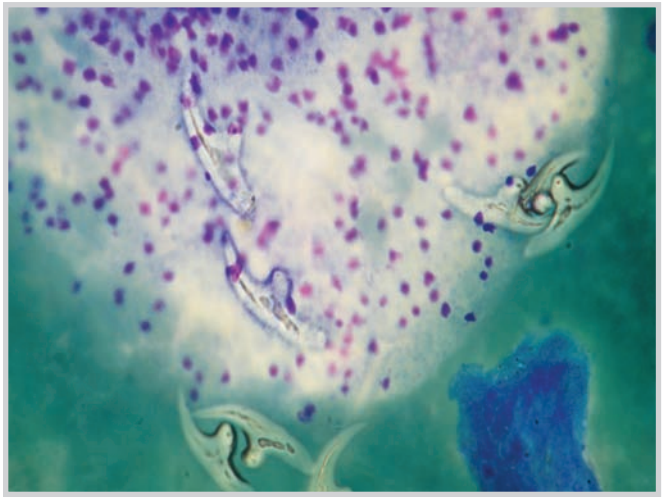

Fig. 30.3A: Multiple hooklets of hydatid cyst in an aspirate from liver (MGG × HP)

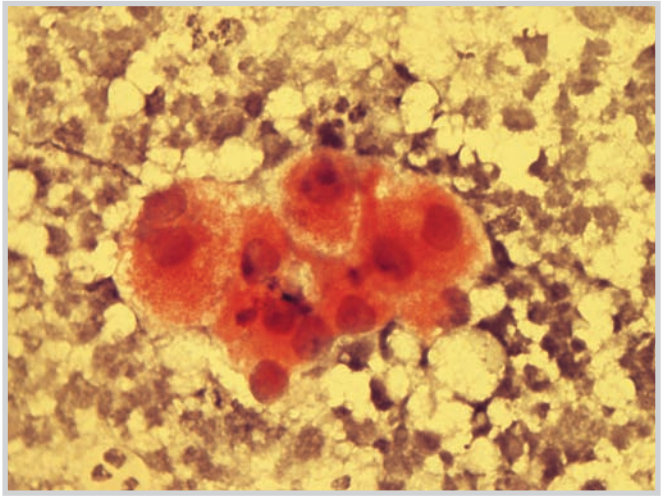

Fig. 30.3B: Pyogenic abscess in liver: Abundant neutrophils and degenerated hepatocytes (H & E × HP)

BOX 30.3 Hydatid cyst

- Laminated membrane
- Scolices
- Hooklets
- Multinucleated giant cells

BOX 30.4 Pyogenic abscess

- Polymorphs
- Necrotic debris
- Degenerated hepatocytes
- Reactive atypia of hepatocytes

Malignant

- Hepatocellular carcinoma (liver cell carcinoma)
- Intrahepatic cholangiocarcinoma
- Peripheral bile duct carcinoma
- Bile duct cystadenocarcinoma
- Combined hepatocellular and cholangiocarcinoma
- Hepatoblastoma
- Undifferentiated carcinoma

Benign Tumor

Focal Nodular Hyperplasia

More than 90% focal nodular hyperplasia (FNH) is seen in females of reproductive age group. Occasionally, it may occur in pediatric age group.[5] FNH possibly results due to hyperplastic response to arterial malformation. CT scan of the liver shows a mass with central stellate scar. The scar enhances in the arterial phase and disappears in the portal venous phase. FNAC of FNH yields a chunk of liver tissue that is difficult to spread on the slide. The smear shows abundant discrete benign hepatocytes along with many fibroblasts in the background (**Box 30.5**). The hepatocytes look completely benign and a diagnosis of FNH is not possible in the cytology smear.

Liver Cell Adenomas

Liver cell adenomas predominantly occur in females under 30 years of age who take oral contraceptive for more than 5 years.[6]

BOX 30.5 **Focal nodular hyperplasia**

- Benign hepatocytes
- Many fibroblasts
- Bile ductular cells

The liver cell adenoma may present with pain abdomen due to rupture of the adenoma into the surrounding tissue. CT scan of liver shows an encapsulated, well circumscribed, low density mass with marked centripetal pattern of enhancement on arterial phase.

Histology of the liver cell adenoma shows only benign hepatocytes without any portal triads.

Cytology (Figs 30.4 and 30.5)

FNAC smear of the liver cell adenoma shows abundant three-dimensional clusters and also dissociated cells. The hepatocytes are normal polygonal in shape with often vacuolated cytoplasm (**Box 30.6**). Nuclei are round and monomorphic in appearance. The background of the smear does not show any bile duct epithelium. The cytological features of liver cell adenoma are nonspecific and the diagnosis is impossible on FNAC smear alone. Clinical history, CT scan findings along with cytology may be helpful in diagnosis of liver cell adenoma.

Differential Diagnosis

- *Hepatocellular carcinoma*
- *Reactive hepatocytes:* The cells in reactive hepatocytes show more nuclear pleomorphism than liver cell adenoma. The absence of bile duct epithelial cells favors liver cell adenoma.

Malignant Tumor

Hepatocellular Carcinoma[7-15]

Hepatocellular carcinoma is the malignant tumor that develops from the hepatocytes. It is one of the most common malignant

BOX 30.6 **Liver cell adenoma**

- Three-dimensional clusters of hepatocytes
- Normal looking hepatocytes
- Mild atypia
- Absence of bile duct epithelial cells

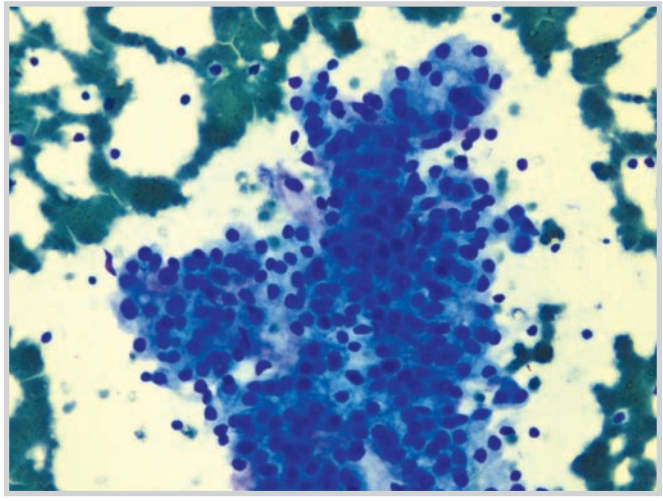

Fig. 30.4: Large cluster of benign looking hepatocytes in fine needle aspiration cytology (FNAC) smear of liver cell adenoma (MGG × MP)

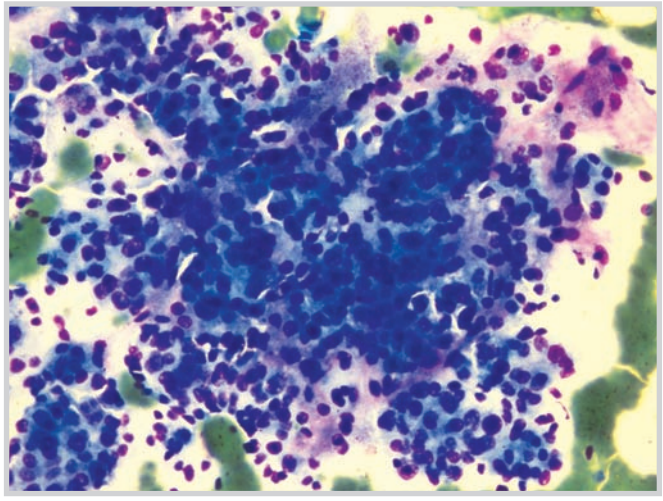

Fig. 30.5: Fine needle aspiration cytology (FNAC) smear of liver cell adenoma showing benign looking hepatocytes (MGG × HP)

tumors in Asia and also a worldwide major public health problem. Males are more commonly affected by HCC than females. It may in any group; however the incidence of HCC is more in 30–40 years of age.

It is also the most common primary malignant tumors of the liver. The common etiologic causes of HCC are:

- Hepatitis B and hepatitis C infection
- Alcoholic cirrhosis
- Dietary ingestion of high dose of aflatoxin
- Long standing use of oral contraceptives
- High dose anabolic steroid
- Iron overload

Out of all these causes, chronic viral infections by *hepatitis B virus* (HBV) and *hepatitis C virus* (HCV) are the most common etiological factors. It has been noted that the individuals with chronic HBV infection carries 100 times more risk of HCC than the uninfected people. HBV vaccination reduces the incidence of HCC.

Cirrhosis of liver is another important risk factor of HCC and almost 90% of HCC develops from cirrhosis. In 60% of HCC cases, alpha-fetoprotein (AFP) level is high. This is one of the important diagnostic markers of HCC. A significantly raised level of AFP level or continuous rising level of the same suggests the possibility of HCC.

The patient of HCC usually presents with generalized weakness, malaise and pain in abdomen. Physical examination of the patients shows hepatomegaly, jaundice and ascities.

Biochemical features: The patients of HCC may show rise of liver enzymes such as aspartate amino transferase, alanine aminotransferase, alkaline phosphatase, gamma-glutamyl-transpeptidase, and bilirubin. These raised enzymes depend on the underlying liver disease and damage. They are non specific for HCC. AFP level is also raised in HCC. Serum AFP level more than 500 ng/ml is highly suggestive of HCC.

Imaging study: Various radiological imaging studies are helpful for the detection of HCC such as USG, CT scan, magnetic resonance imaging (MRI) and color Doppler Ultrasonography.

*Cytology (**Figs 30.6** to **30.13**):* FNAC smear of the liver should be thoroughly studied in low power magnification of the microscope (**Box 30.7**). The smears should be studied for:

- Pattern of the spread of cells
- Individual tumor cells
- Background hepatocytes or other evidences.

Smears with hypercellularity and regularly arranged cells usually indicate the possibility of HCC.

The tumor cells are arranged in following pattern:

- Trabecular: Trabecular pattern is characterized by column of three to four cells and surrounded by endothelial cells.
- Acinar or glandular: The acinar pattern shows a central lumen surrounded by five to eight cells.
- Thin cords: One to two cells thick rows of cells.

The single most important feature of HCC is that the tumor cells resemble hepatocytes. The individual tumor cells are large polygonal with well-defined cytoplasmic border. The nuclei show mild to severe degree of pleomorphism with large prominent macronucleoli. The nuclear membrane is well-defined, irregular and often shows folds and deep indentations. The nuclear

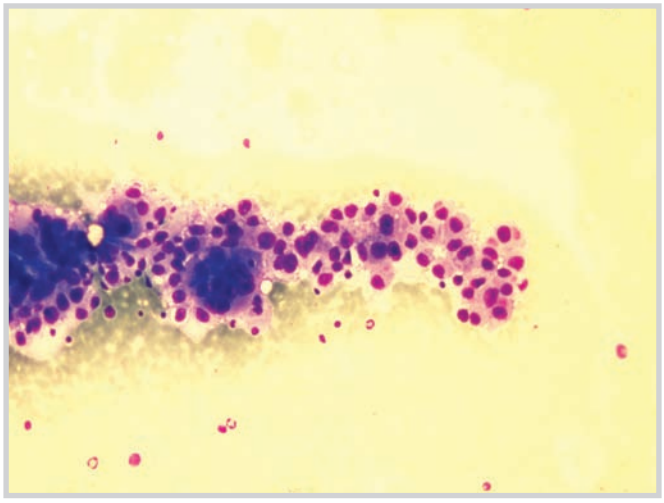

Fig. 30.6A: Hepatocellular carcinoma. The cells are arranged in long cord (MGG × MP)

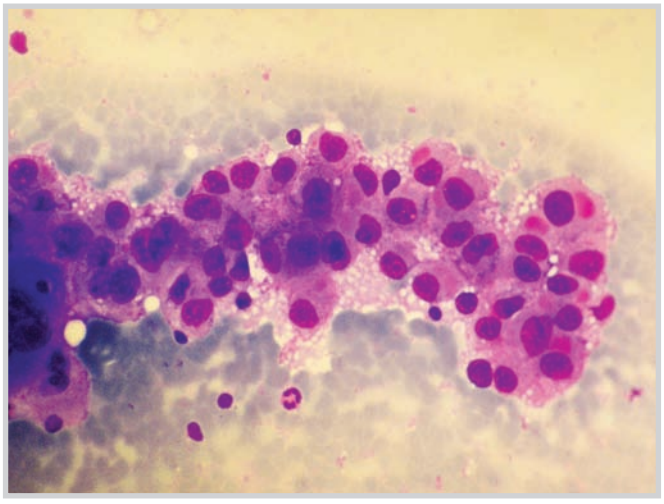

Fig. 30.6B: Hepatocellular carcinoma. Higher magnification of the trabecular arrangement of cells (MGG × HP)

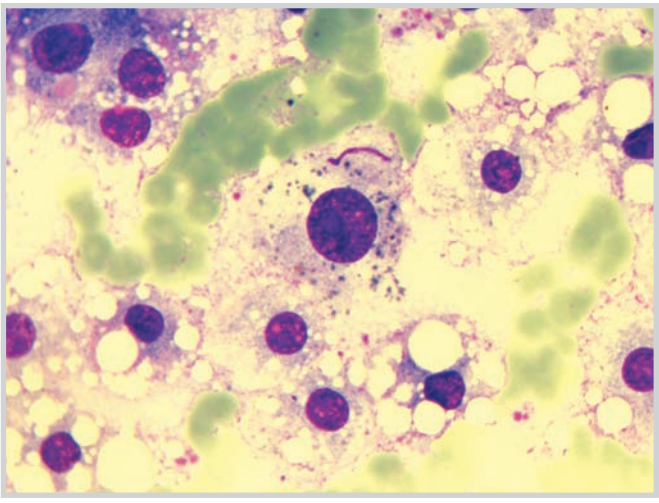

Fig. 30.7: Hepatocellular carcinoma: Bile within the malignant hepatocytes (MGG × HP)

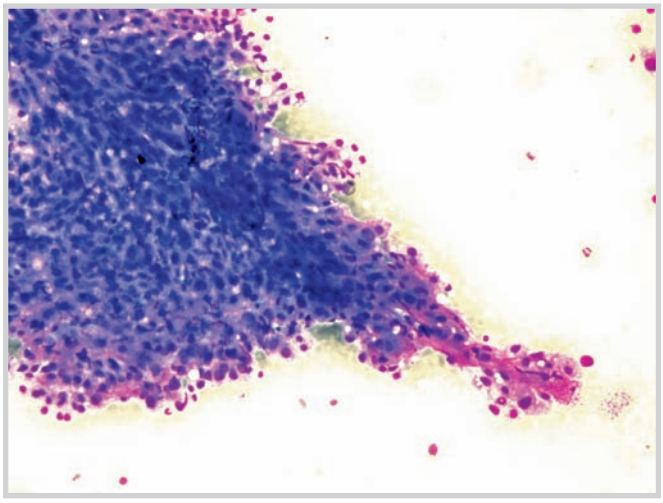

Fig. 30.8A: Hepatocellular carcinoma: Transgressing capillaries within the hepatocytes (MGG × MP)

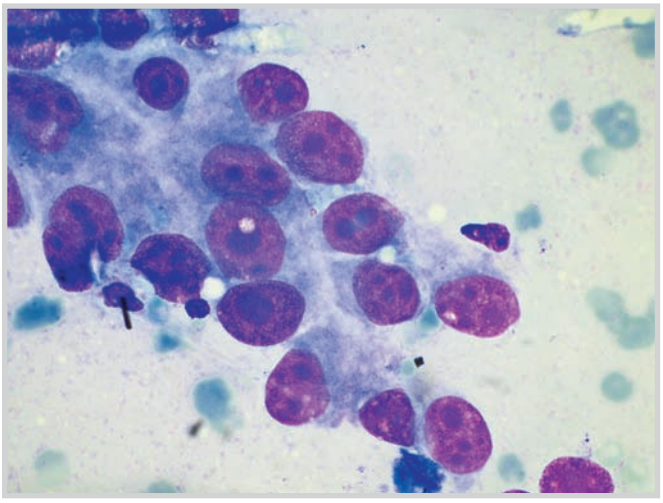

Fig. 30.9: Hepatocellular carcinoma: Higher magnification showing large cells resembling hepatocytes. The nuclei are large, pleomorphic and show multiple prominent nucleoli [May-Grünwald-Giemsa (MGG) stain × oil immersion (OI)]

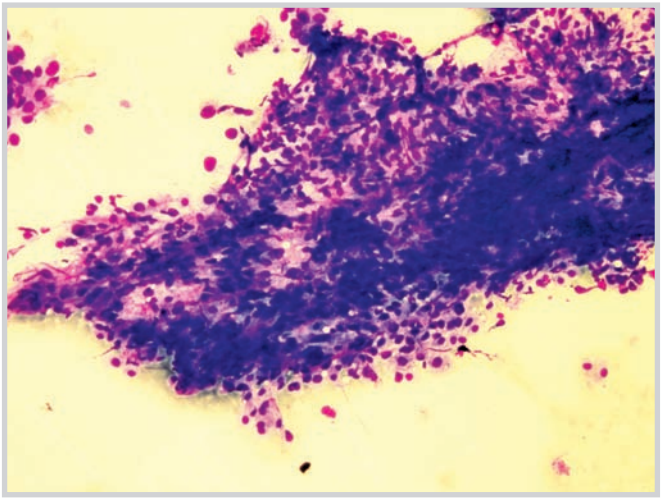

Fig. 30.8B: Hepatocellular carcinoma: In other area long thin capillaries within the malignant cell clusters (MGG × MP)

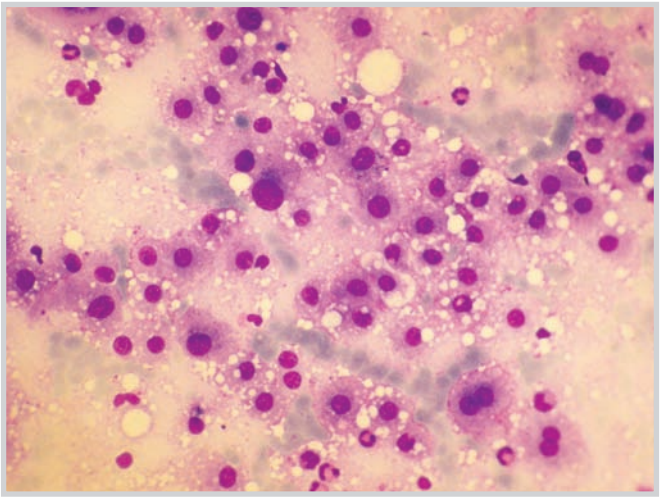

Fig. 30.10: Hepatocellular carcinoma: Well differentiated hepatocellular carcinoma resembles normal hepatocytes (MGG × MP)

chromatin is fine granular. Intranuclear cytoplasmic inclusions may also be seen in HCC and they are not specific.

Intracytoplasmic pale hyaline globules resembling Mallory hyaline bodies and vacuoles containing fat and glycogen may also be noted. The presence of intracytoplasmic bile pigments is found in almost one-third of HCC cases and this is specific for the diagnosis of HCC.

Transgressing thin capillaries lined by the endothelial cells within the large clusters of tumor cells are also noted. Uncommonly, pseudo acini are seen with central dark brown blobs of bile. The cytology smears also show many atypical stripped bare nuclei that are similar to the nuclei present in intact hepatocytes. Isolated tumor giant cells may also be noted.

Reactive or regenerative hepatocytes may often show nuclear enlargement and pleomorphism. Bi- and multinucleated hepatocytes are also seen. The differentiating cytological features of HCC from reactive hepatocytes are abundant cellularity, trabecular pattern of cells, moderate to marked nuclear atypia, stripped atypical nuclei and macronucleoli.

On FNAC, it is difficult to differentiate a metastatic adenocarcinoma from the HCC. However, certain cytological features such as polygonal cells resembling hepatocytes, trabecular pattern of cells, background stripped nuclei, eosinophilic granular cytoplasm and intracellular bile pigments favor a diagnosis of HCC rather than metastatic adenocarcinoma (**Table 30.2**).

At times, it is difficult to differentiate liver cell adenoma from HCC. Both the conditions may show abundant cellularity and nuclear atypia. However, clinical history of cirrhosis raised AFP level, absence of history of steroid intake along with the cytological features of trabecular arrangement of cell surrounded by endothelial lining, stripped atypical cell, and significant nuclear atypia favor a diagnosis of HCC.

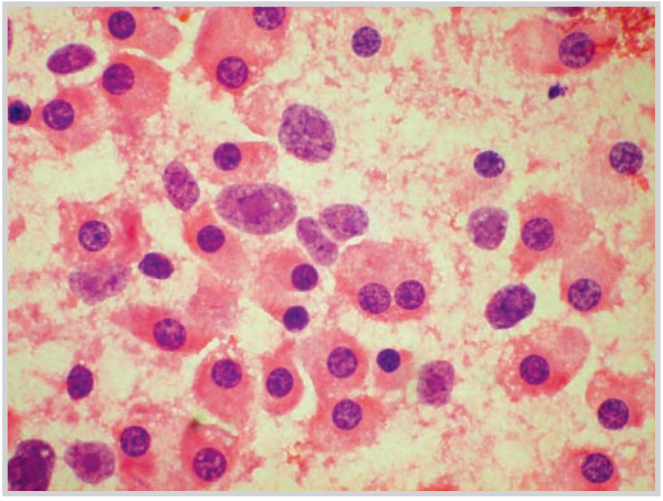

Fig. 30.11: Well-differentiated hepatocellular carcinoma: Polyhedral malignant cells with moderately pleomorphic nuclei having macronucleoli (H & E × OI)

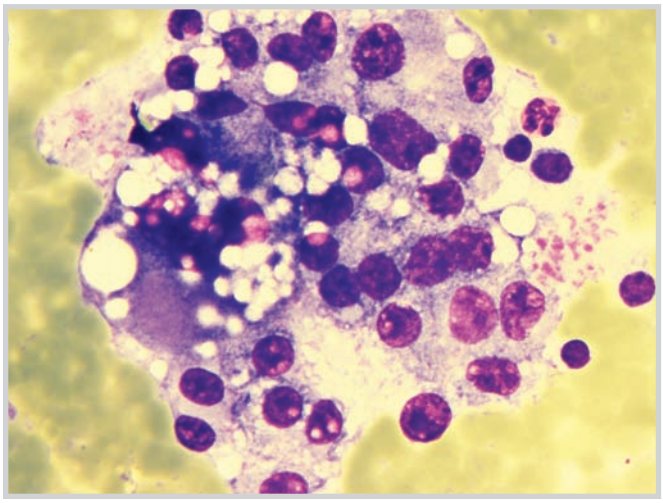

Fig. 30.13: Clear cell type hepatocellular carcinoma: Cells with marked clear vacuolated cytoplasm (MGG × HP)

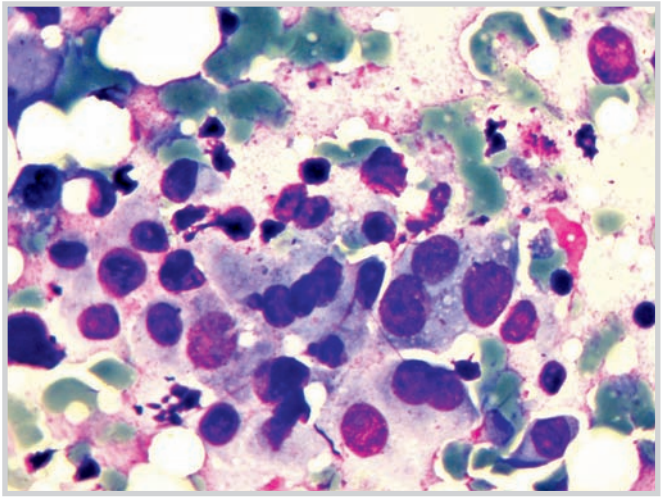

Fig. 30.12: Poorly differentiated hepatocellular carcinoma: Large moderately pleomorphic cells (MGG × HP)

BOX 30.7 Hepatocellular carcinoma

- Hypercellular
- Trabecular, acinar, cord of cells
- Polygonal malignant cell resembling hepatocytes
- Well-defined cell margin
- Atypical bare nuclei
- Transgressing capillaries within clusters
- Enlarge nuclei
- Pleomorphic
- Macronucleoli
- Intracytoplasmic bile pigments
- Intracytoplasmic eosinophilic globules

Immunocytochemistry: Positive for alpha-fetoprotein, Hep Par 1, Glypican-3, polyclonal carcinoembryonic antigen (pCEA) and CD10

Variants of HCC

Fibrolamellar Variant of HCC: It is a distinct clinic-pathological entity. Fibrolamellar variant of HCC is a rare variant of HCC that commonly occurs in 2nd and 3rd decade of life. This tumor presents as single nodule and therefore it is surgically resectable. On histology section, the tumor cells are arranged in sheets or small trabeculae. The cells are separated by hyalinized collagen bundles with a characteristic lamellar pattern.

Cytology: Aspiration cytology smears show dyscohesive large polygonal liver cells with abundant dense granular eosinophilic oncocytic cytoplasm. The nuclei are round, monomorphic with coarse chromatin. Cytoplasmic pale bodies are also characteristic of this tumor. Many benign spindle shaped fibroblasts and fragments of collagen are seen in the background.

Clear Cell Variant: The clear cell variant comprises of almost 10% cases of HCC. Here the cells show abundant clear vacuolated cytoplasm due to accumulation of glycogen. Focal clear cell change may also be seen in classical HCC. This case may be difficult to distinguish from metastatic renal cell carcinoma.

Giant Cell Variant of HCC: Pure giant cell HCC is very uncommon. In this tumor, the FNAC smear shows multiple tumor giant cells. The cell may be large mononuclear or multinucleated.

Small cell variant: The cytology smears of this variant of HCC resemble as neuroendocrine tumor. The tumors show dyscohesive or acinar arrangement of small round to oval cells with scanty cytoplasm. Nuclear features of the cells resemble neuroendocrine tumor. Trabecular pattern is absent in this variant.

TABLE 30.2: Differentiating points between hepatocellular carcinoma and metastatic adenocarcinoma

Features	*Hepatocellular carcinoma*	*Metastatic adenocarcinoma*
Pattern of cells	Trabecular, acinar, small cluster	Glandular
Transgressing capillaries	Present	Absent
Associated benign hepatocytes	Usually absent	Present
Bare nuclei	Present	Absent
Cell shape	Polygonal	Columnar
Cytoplasm	Eosinophilic granular	Vacuolated
Macronucleoli	Present	Usually absent
Intracytoplasmic bile pigment	Present	Absent
HbsAg	Positive	Negative
Alpha feto protein level in serum	Raised	Mostly normal
Immunocytochemistry HepPar 1, Glypican-3, pCEA and CD10	Positive	Negative

Abbreviations: Hep Par 1, Human hepatocyte antibody; p-CEA, polyclonal carcinoembryonic antigen

Spindle cell variant: The pure form of spindle cell variant is uncommon. The cytology smear shows spindle-shaped hepatocytes in small clusters along with many giant hepatocytes. The individual cells show spindle-shaped nuclei with moderate nuclear pleomorphism. If the smear contains predominantly spindle cells then it may be difficult to differentiate this tumor from a metastatic spindle cell sarcoma such as fibrosarcoma or leiomyosarcoma.

Hepatocellular Carcinoma with Fatty Change: In this variant, the smears show malignant hepatocytes with remarkable fatty changes. This variant of HCC may be mistaken for fatty change in the liver. Poorly-differentiated HCC with fatty change may be mistaken for metastatic liposarcoma.

Immunocytochemistry and Serological Markers of HCC (Box 30.8):

Immunocytochemistry or serological markers are helpful for confirmation of the primary malignancy in liver or the histogenetic origin of the tumor.

Alpha-fetoprotein: This is one of the commonly used markers for the diagnosis of HCC. This protein is secreted by the neoplastic liver cells and the normal hepatocytes do not express AFP.

BOX 30.8 Immunocytochemical markers of HCC

Alpha-fetoprotein (AFP)
- Cytoplasmic positivity
- Neoplastic hepatocytes
- Low sensitivity, good specificity
- Confirmation of HCC

Hepatocyte (Hep Par1)
- A hepatocyte mitochondrial epitope
- Cytoplasmic granular positivity
- High sensitivity (100%) in well differentiated HCC
- 90% specific for HCC
- Distinguishes HCC from cholangiocarcinoma and metastatic adenocarcinoma

Glycipan 3
- Sulfate proteoglycan on cell membrane
- Very high sensitivity and specificity
- Only malignant hepatocytes are positive

Polyclonal carcinoembryonic antigen (p-CEA)
- Stains bile canaliculi and bile duct epithelium
- Low sensitivity and high specificity
- Distinguishes HCC from metastatic adenocarcinoma

The sensitivity of this marker is near about 50%.[16] The specificity of AFP is relatively high. However, the raised AFP level may be noted in malignancies other than HCC such as gastric adenocarcinoma and large cell neuroendocrine carcinoma.[17] There is a growing tendency to drop this marker from the diagnostic panel of HCC.

Hepatocyte (Hep Par 1): Human hepatocyte antibody (Hep Par 1) recognizes a hepatocyte mitochondrial epitope. It shows cytoplasmic granular positivity. Various studies have shown that Hep Par 1 is relatively sensitive, specific and very helpful in detecting HCC, especially the well-differentiated HCC.[18,19]

Unfortunately, its sensitivity drops remarkably in poorly differentiated HCC. Hep Par1 staining may also be seen in tumors other than HCC such as gastric, pulmonary, colon, breast, and pancreatic adenocarcinoma.[20]

Polyclonal carcinoembryonic antigen (p-CEA): The presence of intercellular canaliculi is one of the important diagnostic features of HCC. p-CEA stains bile canaliculi and duct but not the hepatocytes. In well-differentiated HCC, this can be demonstrated as long linear pattern, whereas in poorly-differentiated HCC it is thick and short.

CD 10: This is expressed in normal and neoplastic liver cells and therefore helps in differentiation HCC from metastatic carcinoma. It shows similar canalicular pattern to p-CEA.

Glycipan-3: This is a member of the sulfate proteoglycans family. The glypican-3 gene is located on xq26.1. Glycipan 3 is expressed in the cell membrane and plays an important role in cell growth, migration and differentiation. Immunocytochemistry displays cytoplasmic reaction with membranous accentuation. It is a highly sensitive and specific marker of HCC and is more useful than Hep Par 1 in detecting poorly differentiated HCC. Glycipan 3 is especially useful in detecting mass lesions of HCC from

other benign and premalignant liver nodules such as liver cell adenoma, dysplastic nodules, and FNH nodules.

Differential Diagnosis
- Reactive hepatocytes
- Adenocarcinoma
- Liver cell adenoma

Cholangiocarcinoma

This is far less common than HCC. The tumor arises from the bile duct epithelium, intrahepatic bile ducts or extra-hepatic biliary tree. Cholangiocarcinoma is often associated with *Clonorchis sinensis* infestation, hemochromatosis, Thorotrast injection and chronic ulcerative colitis.[21] The patients usually present with weakness, malaise and obstructive jaundice.

Cytology

FNAC smear shows small clusters and microglandular arrangement of cells. The individual cells are cuboidal to columnar with scanty cytoplasm and resemble atypical bile duct epithelial cells (**Box 30.9**). Nuclei are mildly enlarged with prominent nucleoli. Occasional cells show vacuoles in the cytoplasm. On FNAC smear, cholangiocarcinoma cannot be distinguished from metastatic pancreatic adenocarcinoma. The cells of cholangiocarcinoma are positive for cytokeratin 7 (CK 7), CK19, epithelial membrane antigen (EMA), and diffusely positive for p-CEA. In contrast HCC cells are positive for CK8 and CK18.[22]

Differential Diagnosis
- Metastatic adenocarcinoma

Hepatoblastoma[23-26]

Hepatoblastoma is the tumor of the children and young adults and 90% of this tumor occurs before 5 years of age. This is a relatively rare tumor. However, hepatoblastoma is the most common malignant tumor of liver in children. The tumor is usually solitary space occupying lesion in liver. The children with hepatoblastoma are presented with a large bulging abdominal mass, hepatomegaly with abdominal swelling, and fever.

Cytology (Figs 30.14 to 30.16)

Hepatoblastoma may be of three types (**Box 30.10**):

1. *Anaplastic:* The anaplastic type of hepatoblastoma shows small round cells with scanty cytoplasm and round nucleus. Anaplastic type of hepatoblastoma may cause diagnostic

BOX 30.9 Cholangiocarcinoma

- Small clusters and sheets
- Small cuboidal to columnar cells
- Round hyperchromatic nuclei
- Scanty cytoplasm.
- Normal alpha fetoprotein level.

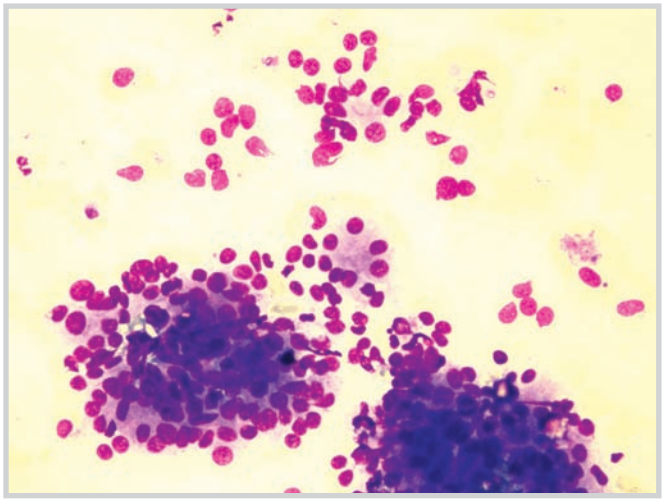

Fig. 30.14: Hepatoblastoma: Round to oval cells in cluster and ill-defined acini (MGG × MP)

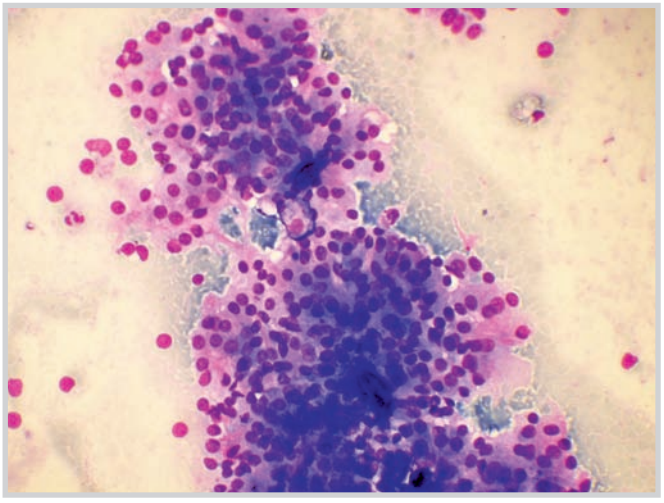

Fig. 30.15: Hepatoblastoma: Cell with moderate amount of cytoplasm and monomorphic nuclei (MGG × MP)

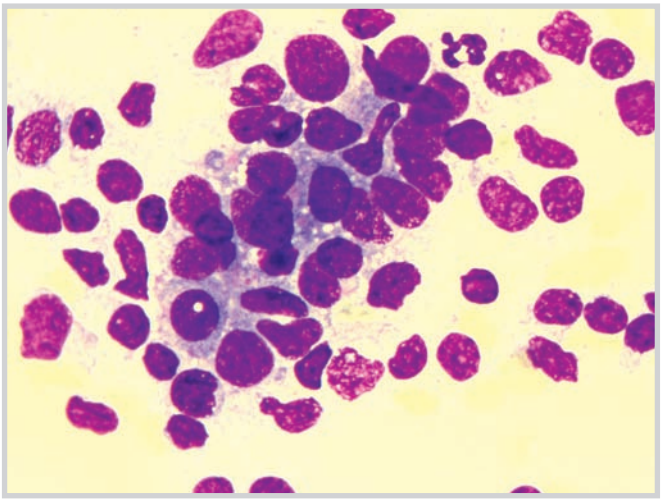

Fig. 30.16: Hepatoblastoma: Higher magnification showing cells with moderate cytoplasm and fine nuclear chromatin (H & E × OI)

BOX 30.10 Hepatoblastoma

Anaplastic
- Small round cells

Embryonal
- Sheets, clusters and rosettes
- Larger than anaplastic type
- Immature cells with high nucleus/cytoplasm ratio (N/C) ratio, irregular hyperchromatic nuclei

Fetal
- Larger than the embryonal cells
- Round to polygonal with well-defined cytoplasmic border
- Round nuclei
- Minimal nuclear pleomorphism
- Finely granular chromatin
- Mesenchymal components: osteoid material or skeletal muscle
- Extramedullary hematopoiesis

difficulties with other small round cell tumors such as neuroblastoma and rhabdomyosarcomas.

2. *Embryonal:* The cells of embryonal type are arranged in sheets, clusters and rosette-like structures. The individual cells are larger than the small round cells of anaplastic type. They are immature with high N/C ratio, irregular hyperchromatic nuclei. Nuclear chromatin is dense with single to multiple nucleoli. The cytoplasm is scanty to moderate in amount. The cells lack the differentiation of hepatocytes.
3. *Fetal:* Fetal cells resemble fetal hepatocytes and form well-formed acini. These cells are larger than the embryonal cells. The cells are round to polygonal with well-defined cytoplasmic border and high N/C ratio. Nuclei are round with minimal nuclear pleomorphism and finely granular chromatin. These cells may contain bile pigment. Many traversing capillaries may be seen with perivascular arrangement of tumor cells resembling sinusoids. Mesenchymal components are seen in the form of clusters of spindle cells with elongated nuclei. Osteoid material or skeletal muscle differentiation may also be seen. Extramdeullary hematopoiesis in the form of nucleated RBC, megakaryocytes and myeloid series of cells are frequently noted. Fetal type of hepatoblastoma may be confused with HCC. The presence of metaplastic elements and extramedullary hematopoiesis favor a diagnosis of hepatoblastoma.

Differential Diagnosis
- Hepatocellular carcinoma
- Other small round cell tumor

Primary Sarcomas

Angiosarcoma

This is the most common sarcoma of liver. However angiosarcoma is a rare tumor. The tumor occurs in 6th to 7th decade. The exact etiological cause of this tumor is unknown in majority of the cases. However, this tumor is often associated with prior administration of Thorotrast (a radiographic dye), exposure to vinyl chloride monomer and use of androgenic-anabolic steroids.

BOX 30.11 Angiosarcoma

- Cohesive clusters and discrete cells
- Oval to spindle-shaped nuclei
- Moderate nuclear pleomorphism
- Tumor giant cells
- Cytoplasmic vacuolations

Immunostain: CD31, CD34

BOX 30.12 Epithelioid hemangioendothelioma

- Hypocellular
- Large pleomorphic cells
- Tadpole like cells
- Abundant lacy cytoplasm
- Pleomorphic nuclei with folding
- Intranuclear cytoplasmic inclusions
- Bi- and multinucleated giant cells

Immunostain: CD31, CD34

Cytology

The cytology smear shows discrete and tight cohesive clusters of malignant spindle cells (**Box 30.11**). The nuclei are oval to elongated with blunt ends having moderate pleomorphism. The cytoplasm shows fine vacuolations and intracytoplasmic lumen.

The cells of angiosarcoma are positive for CD31 and CD34 immunostaining.

Epithelioid Hemangioendothelioma

This is also a rare tumor in the liver arising from vascular endothelial cells. This is less aggressive tumor than angiosarcoma.

Cytology

FNAC smears are usually paucicellular. There are loose clusters and discrete large cells with abundant lacy cytoplasm (**Box 30.12**). Many of the tumor cells have tail like elongated cytoplasm. The nuclei are moderately pleomorphic with prominent nuclear folding and distinct nucleoli. Intranuclear cytoplasmic inclusions are also seen. In addition, there may be many bi- and multinucleated giant cells.[27]

METASTATIC MALIGNANCIES

Liver is a highly vascular organ and therefore commonly harbors metastatic tumors. In our institution, more than 90% of FNAC of liver mass are diagnosed as metastatic carcinoma. The common sources of metastasis are from gastrointestinal tract, breast, lung, kidney, gallbladder and pancreas. Malignant tumors from the

adjacent organs such as gallbladder, pancreas and stomach may directly invade to the liver parenchyma. Sarcomas, melanomas and lymphomas may also infiltrate in the liver. At times, the metastatic tumors may simulate HCC and pose diagnostic problems.

Cytology (Figs 30.17 to 30.22)

Cytology features of metastatic malignancies depend on the morphology of the primary tumor (**Box 30.13**). Therefore, a thorough look of the smear by low power magnification is essential. The study of cell arrangement, overall cell morphology and background material are helpful.

Adenocarcinoma

FNAC smear of metastatic carcinoma shows clusters and glandular arrangement of cuboidal to columnar epithelial cells. The recognition of such foreign cells is very important to distinguish them from the cells of HCC.

The cytological features depend on the type of the metastatic lesions. In case of metastatic adenocarcinoma, the individual cells have scanty to moderately vacuolated cytoplasm. Nuclei are round, moderately pleomorphic having prominent nucleoli. Benign hepatocytes may be admixed with the malignant cells.

Squamous Cell Carcinoma

FNAC of metastatic squamous cell carcinoma shows oval to polyhedral cells with enlarged hyperchromatic nuclei. Cytoplasmic orangeophilia in Papanicloaou's staining is characteristic of squamous cells. The smears also show fiber cells and tadpole-like cells.

Small Cell Carcinoma

Cytology smears of small cell carcinoma show abundant discrete and loose clusters of small cells slightly larger than lymphocytes. The cells show nuclear molding. Individual cells have scanty cytoplasm and round hyperchromatic nuclei with inconspicuous to absent nucleoli. Crushing artifact may also be seen in the background.

Malignant Melanoma

FNAC smears of melanoma may present with variable morphology. The cells are usually round to oval. However, spindle cells may also be seen. The individual cells show moderate to marked nuclear enlargement and pleomorphism. Nucleoli are large and variable sized. Cytoplasmic dark black melanin pigment indicates the diagnosis of melanoma.

Metastatic Neuroendocrine Tumors

Neuroendocrine tumors from pancreas or other part of the gastrointestinal tract often metastasize in the liver. The malignant

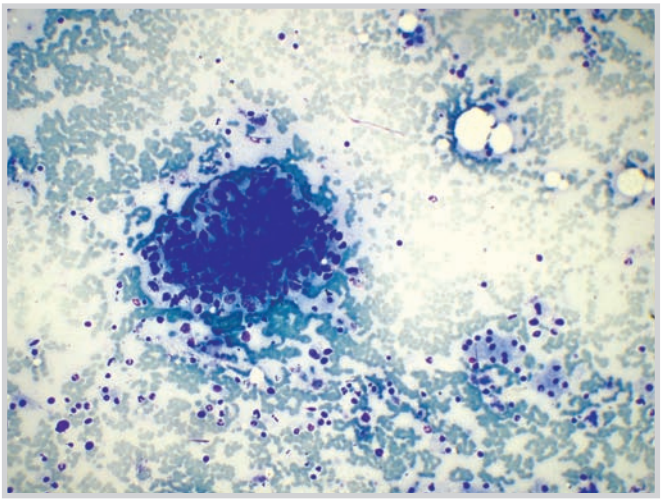

Fig. 30.17A: Metastatic adenocarcinoma: Cluster of malignant cells with background benign hepatocytes (MGG × MP)

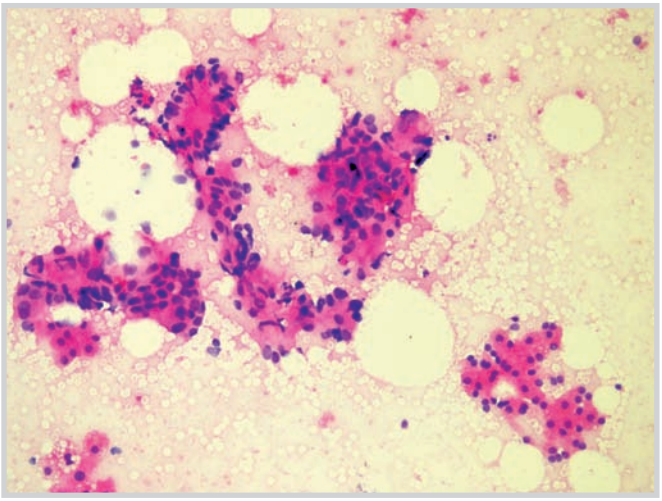

Fig. 30.17B: Metastatic adenocarcinoma: Malignant cells with gland formation along with benign hepatocytes (H & E × MP)

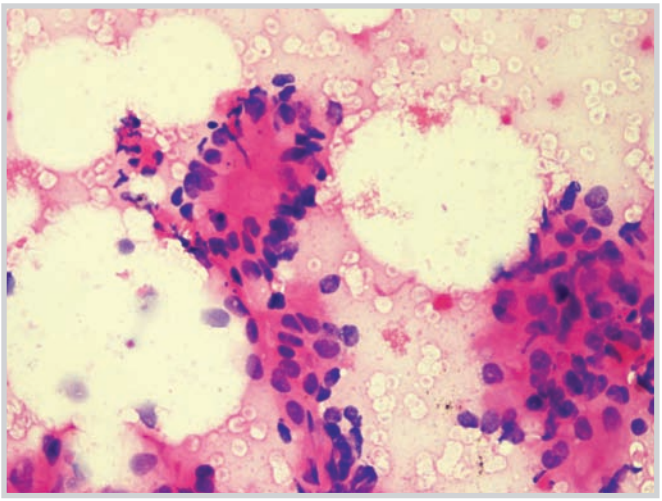

Fig. 30.17C: Metastatic adenocarcinoma: Higher magnification showing glandular differentiation (H & E × HP)

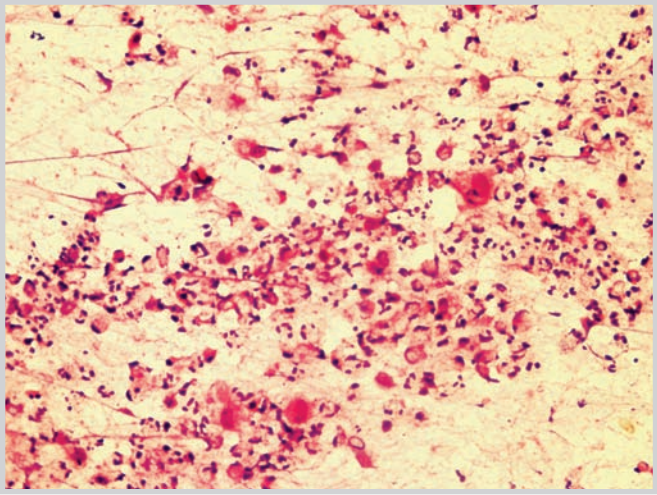

Fig. 30.18: Metastatic signet ring cell carcinoma: Discrete signet ring cells with abundant vacuolated cytoplasm and eccentric signet ring-like nuclei. The primary tumor was in stomach (H & E × MP)

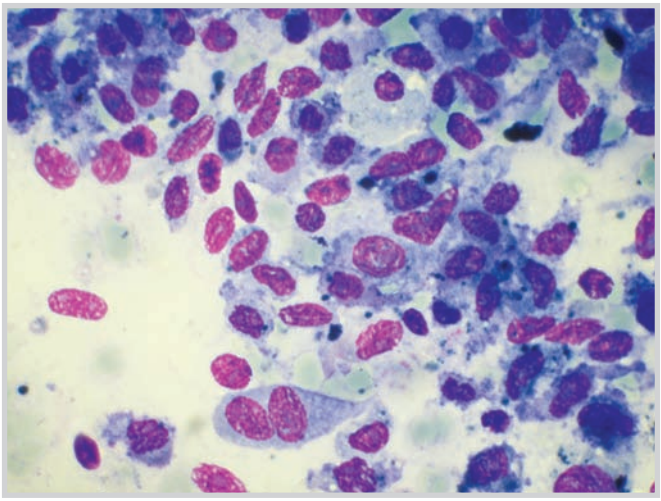

Fig. 30.19: Metastatic malignant melanoma: Discrete melanoma cells with dark black melanin pigment within the cytoplasm (MGG × OI)

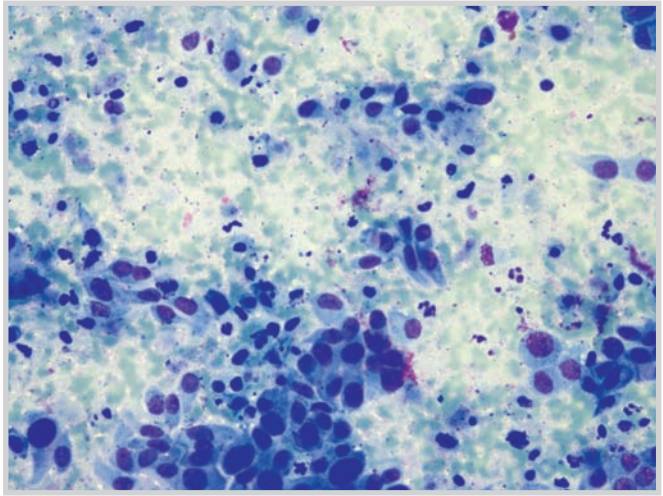

Fig. 30.20: Metastatic transitional cell carcinoma: Discrete cells with elongated cytoplasm and basally placed nuclei (MGG × HP)

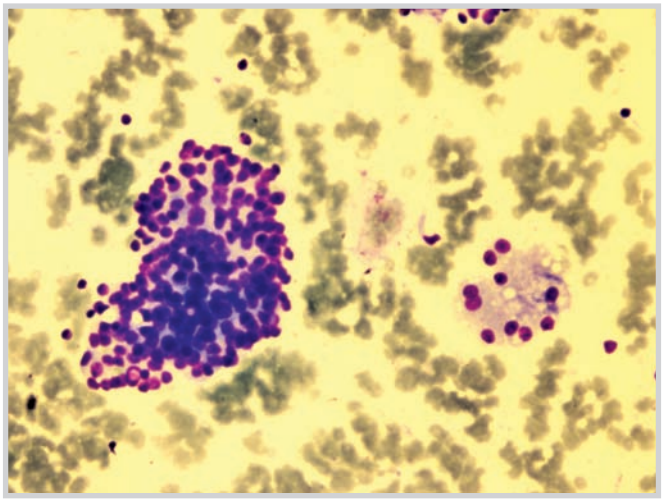

Fig. 30.21: Metastatic neuroendocrine tumor: Loose clusters of round monomorphic cells along with benign hepatocytes in a case of pancreatic tumor (MGG × MP)

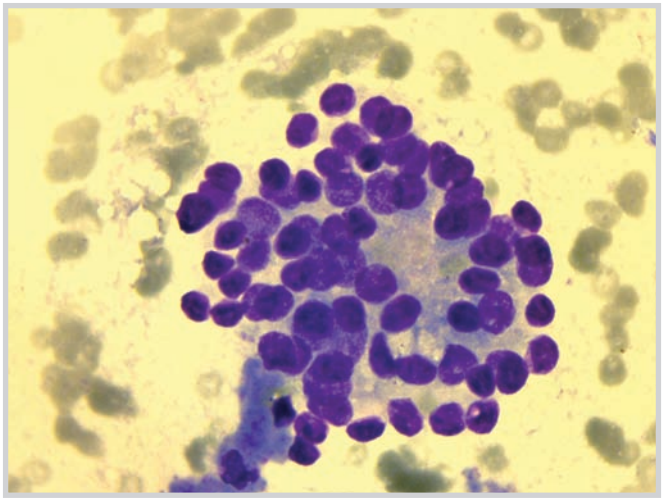

Fig. 30.22: Metastatic neuroendocrine tumor: High-powered view show rosette-like structure in the same smear (MGG × MP)

BOX 30.13 **Metastatic malignancies in liver**

- Cells look foreign to liver
- Glandular arrangement
- Cuboidal to columnar cells
- Benign hepatocytes
- Round moderately pleomorphic nuclei with prominent nucleoli
- Normal alpha-fetoprotein level

cells are usually monomorphic with round regular nuclei and scanty cytoplasm. Pseudorosettes are also seen.

Differential Diagnosis

- Hepatocellular carcinoma

Malignant Lymphoma

Primary lymphoma of the liver is rare and it is defined as an extranodal lymphoma where the main bulk of the tumor is localized in the liver. However, the *secondary lymphoma* is not an uncommon entity and 50–60% cases of non-Hodgkin's lymphoma (NHL) involve liver in advanced stage. The primary lymphomas of liver include diffuse large B cell lymphoma (DLBCL) and Burkitt's lymphoma. Occasionally, mucosal-associated lymphomas (MALTOMAs) of liver are also described.[4] As the liver involvement is patchy and diffuse so FNAC may miss such lesions.

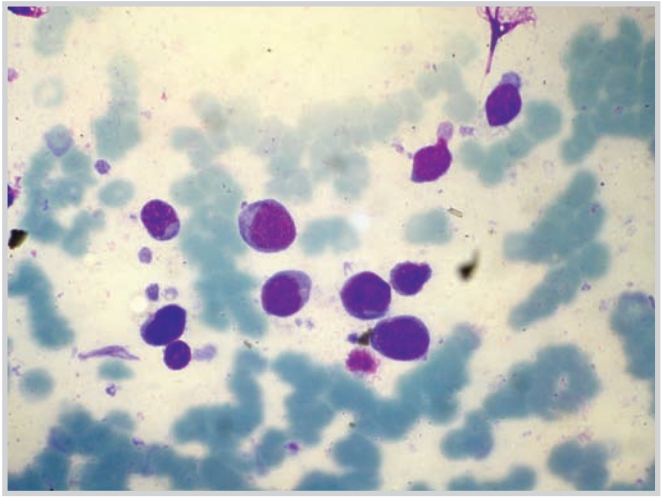

Fig. 30.23: Non-Hodgkin's lymphoma (NHL) of spleen: Discrete immature lymphoid cells in spleen (MGG × OI)

SPLEEN

Fine needle aspiration cytology of the spleen is mainly indicated in diagnosis of metastatic carcinoma and lymphomas that cause space occupying lesion. Occasionally FNAC of spleen is done in diffuse infiltrative process such as storage disorder or infective process such as leishmaniasis. Spleen is an extremely vascular organ and therefore FNAC of this organ is not free from complications. The major complication of FNAC is hemorrhage followed by shock. If small bore needle is used (22 gauze) and the procedure is rapidly done under USG guidance, then no serious complications are encountered. In our institution, we routinely perform FNAC of the spleen and fortunately we did not face any serious complications.

Non-neoplastic Process

Granulomatous Infection

Tuberculosis is one of the most common causes of granulomatous inflammation of spleen. Multiple epithelioid cell granulomas, multinucleated giant cells and necrosis are common findings.

Sarcoidosis of spleen shows noncaseating granulomatous inflammation and on FNAC smear it is not possible to diagnose sarcoidosis.

Various fungal infections may show granulomatous inflammation. The smears show typical fungal profile along with epithelioid cell granuloma, eosinophils and multinucleated giant cells.

Storage Disorders

Fine needle aspiration cytology smears of Gaucher's disease shows classical large histiocytes with abundant foamy cytoplasm. Biochemical analysis is needed for the confirmation of Niemann-Pick disease.

Neoplasm

Lymphoma

The cytomorphology and the diagnostic criteria of NHL and Hodgkin's lymphoma (HL) of the spleen are essentially same as that of noted in lymph nodes (**Fig. 30.23**). At times low grade lymphoma or follicular lymphomas are difficult to diagnose on the FNAC of spleen.

Sarcomas

Rarely angiosarcoma may occur in spleen. Cytomorphology of this tumor is essentially same as has been described before in connection with liver.

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CHAPTER 31

Pancreas

Chapter Contents

PANCREAS

This is the retroperitoneal organs with both endocrine and exocrine glands. Most of the space occupying lesions in the pancreas are malignant in nature. In addition, various imaging technologies such as computed tomography (CT) scan, ultrasonography (USG) and magnetic resonance imaging (MRI) provide important information on the location, extent and nature of the lesion. However, many times a chronic inflammatory reaction in the pancreas may be very difficult to distinguish from a malignant tumor, even after open surgical exploration. Fine needle aspiration cytology (FNAC) is very helpful in confirming or excluding a malignant tumor of pancreas. FNAC, in fact, has little contribution in the early diagnosis of pancreatic carcinoma. It just helps to confirm the malignant nature of the lesion and prevents unnecessary surgical exploration. Rarely, FNAC diagnosis of a benign lesion may help the patient immensely. In case of pancreatic pseudocyst, FNAC is both a diagnostic and therapeutic procedure. Occasionally, during endoscopic guided FNAC of the pancreas, the stent can be inserted in the stenotic duct in the same sitting after FNAC of the tumor.

Fine needle aspiration cytology of pancreas can be done by (1) USG guidance, (2) CT scan guidance, (3) Endoscopic USG-guidance (EUS-FNAC). The material from the lesion can also be procured at the time of endoscopic retrograde cholangiopancreatography (ERCP) or brushing the biliary tract.[1-3]

Transabdominal USG guidance FNAC is easy to perform and a rapid technique. The tip of the needle is also visible in real time. The major disadvantage is the reduction of visibility in case of fatty patient and by the abdominal bowel gas. In contrast, CT guided FNAC provides better visualization than USG.

Endoscopic USG guidance-fine needle aspiration cytology (EUS-FNAC) is a relative newer technique that can be performed by endoscopically by inserting high frequency ultrasound probe and needle within the gastrointestinal tract.[4] EUS-FNAC is a highly accurate clinical diagnostic test for the detection and grading of malignant tumor. EUS-FNAC is a less costly, less risky, and less invasive strategy of getting material from the small pancreatic lesion.

Echoendoscope has a transducer attached in front of the optic lens. A *radial scanning* echoendoscope gives a 360° view and a *liner array* echoendoscope produces an oblique 100° range real time view parallel to the echoendoscope. The later one is preferable for FNAC. Tumor as less as 5 mm diameter can be detected by echoendoscopy and FNAC can be done by this technique.

Overall sensitivity and specificity of pancreatic FNAC are as high as 90% and 100%, respectively.[5-9] The false negative cytology is due to sampling error. This may happen in very small lesion, fibrotic lesion or sampling from the necrotic area of the tumor. Interpretation error giving rise to false negative cytology, mainly occurs in well-differentiated pancreatic carcinoma. False positive cytology rarely happens in pancreatic FNAC.

The complications of FNAC are rare if thin needle of 21 or 22 gauze is used. Minor complications such as pain, small hemorrhage and vasovagal attack are known. The major complications of pancreatic FNAC include massive hemorrhage and pancreatitis. We routinely use FNAC of pancreas in our institution and have never faced severe pancreatitis after FNAC.

NORMAL ANATOMY AND HISTOLOGY

Pancreas is situated in upper part of the abdomen behind the peritoneum. It has four parts: (1) uncinate process, (2) head, (3) neck and (4) tail part. Pancreas is composed of both exocrine and endocrine glands. The exocrine part of the pancreas consists of lobules that are made of bunches of acini. The acini are connected to the small ducts and these ducts communicate with each other to form larger ducts. The acini are lined by cells with abundant granular cytoplasm and small monomorphic round nuclei. The ducts are lined by flattened to cuboidal epithelium depending on the size of the ducts. The acini secrete many digestive enzymes that include pancreatic amylase, pancreatic lipase, trypsinogen, chymotrypsinogen, procarboxypeptidase, elastase, etc. The endocrine pancreas is composed of spherical aggregates of endocrine cells that are commonly known as Islets of Langerhans. These are small polyhedral cell arranged in small nests. The endocrine cells are dispersed throughout the entire pancreas, however they are more concentrated in body and tail region of the pancreas. There are several types of islet cells based on the hormone production: (1) Alpha cells: secretes glucagon, (2) Beta cells: Insulin, (3) Delta cells: somatostatin, (4) G cell: gastrin, (5) Enterochromaffin cells: 5-hydroxytryptamine and (6) Pancreatic polypeptide cells: Pancreaticpolypeptide.

Normal Cells

Pancreatic acinar cells: Pancreatic acinar cells are usually present in small acinar-like structures or in loose cohesion (**Figs 31.1A** and **B**). The cells are large with indistinct cell border. Cytoplasm of the cell is abundant and granular (**Box 31.1**). The nuclei are round and monomorphic with granular chromatin and inconspicuous nucleoli.

Ductular cells (**Fig. 31.2**): The pancreatic ductular cells are in small clusters and in monolayered sheets. The cells are round with scanty cytoplasm having monomorphic small round nuclei with fine granular chromatin and small nucleoli.

CYSTS OF PANCREAS

Pancreatic cysts may be: acquired or congenital. The acquired cysts may be benign or malignant.

Congenital Cyst

These are benign cysts and rarely found in the pancreas. Benign cysts are commonly associated with inherited polycystic disease. FNAC yields clear fluid and smears are usually paucicellular. Occasional clusters of benign columnar cells are seen.

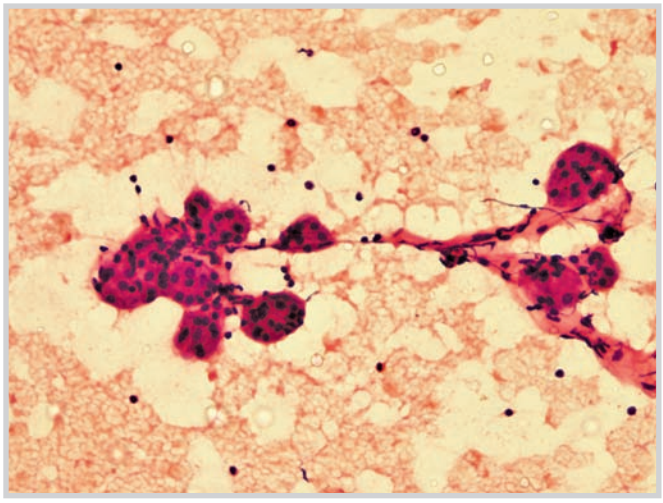

Fig. 31.1A: Pancreatic acinar cells: Multiple clusters of cells with abundant cytoplasm and round central nuclei [hematoxylin and eosin (H & E) stain × medium power (MP)]

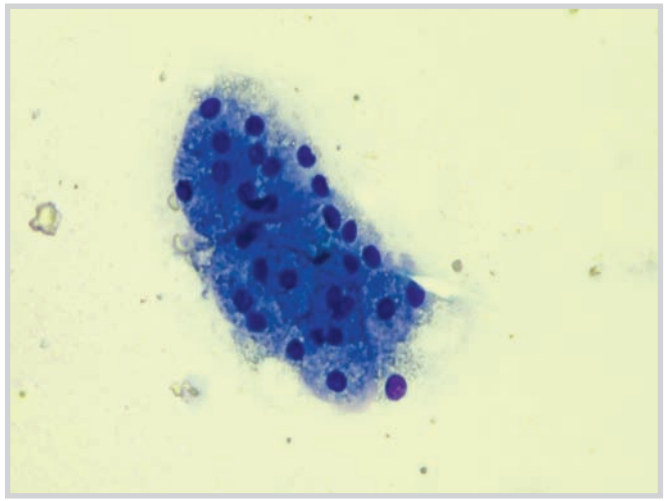

Fig. 31.1B: Pancreatic acinar cells: Abundant granular cytoplasm [May-Grünwald-Giemsa (MGG) stain × high power (HP)]

BOX 31.1 **Pancreatic acinar cells**

- Acinar arrangement
- Ill-defined border
- Abundant granular cytoplasm
- Small round bland nucleus

Acquired Cysts

Pancreatic Pseudocyst

Pseudocyst of the pancreas is a benign acquired cyst. This occurs after an acute bout of pancreatitis or reflux of bile from the biliary tree to pancreatic duct. This causes leakage of bile from the pancreatic duct and accumulation of secretion resulting in

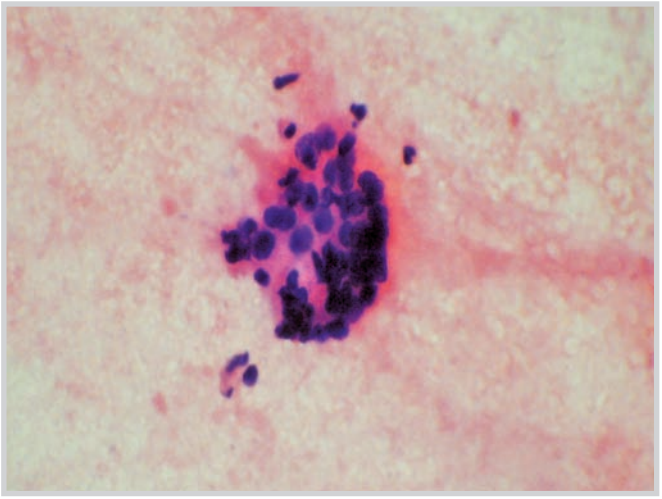

Fig. 31.2: Pancreatic ductular cells: Tight clusters of cells with scanty cytoplasm and round nuclei (H & E × MP)

BOX 31.2 Pseudocyst: Cytology

- Foamy histiocytes
- Polymorphs and lymphocytes
- Fibroblasts
- Reactive mesothelial cells
- Absent epithelial cells

cyst formation. The patient presents with pain abdomen, nausea, vomiting and jaundice.

Cytology

Fine needle aspiration of cytology of pseudocyst has therapeutic value because the evacuation of the cyst gives immediate symptomatic relief to the patient. FNAC from the cyst yields clear fluid (**Box 31.2**). The smear shows foamy macrophages, mixed inflammatory cells and spindle shaped fibroblasts. Characteristically, epithelial cells are absent in the smear. The presence of epithelial cells excludes the possibility of pseudocyst. The histiocytes often show multinucleation. In addition the smear may also show mesothelial cells. At times, these cells show reactive atypia.

NEOPLASTIC LESION OF PANCREAS

World Health Organization (WHO) classified pancreatic neoplasm as epithelial, non-epithelial and metastatic tumors.[10] The simplified version of the classification is shown in **Box 31.3**.

Serous Cystadenoma

Serous cystadenomas are rare tumor and represents only 1% of the pancreatic exocrine tumors. The tumor predominantly occurs in female and mean age of the patient is 66 years. Serous cystadenomas usually presents as large cystic mass located in the body and tail of the pancreas.

BOX 31.3 World Health Organization (WHO) classification of pancreatic tumors

Benign
- Serous cystadenoma
- Mucinous cystadenoma
- Intraductal papillary-mucinous adenoma

Borderline
- Mucinous cystic neoplasm with moderate dysplasia
- Intraductal papillary-mucinous neoplasm with moderate dysplasia
- Solid-pseudopapillary neoplasm

Malignant
- Ductal adenocarcinoma
- Serous cystadenocarcinoma
- Mucinous cystadenocarcinoma
- Intraductal papillary-mucinous carcinoma
- Acinar cell carcinoma
- Acinar cell cystadenocarcinoma
- Mixed acinar-endocrine carcinoma
- Pancreatoblastoma
- Solid-pseudopapillary carcinoma

Nonepithelial tumors

Metastatic tumors

BOX 31.4 Pancreatic serous cystadenoma

- Clear, watery fluid
- Bland looking round to polygonal cells
- Scanty to moderate cytoplasm
- Bland, round monomorphic nuclei

Cytology

Fine needle aspiration cytology of the cyst yields clear thin fluid. The smear shows small clusters and monolayered flat sheets of cells. The cells have scanty to moderate cytoplasm with monomorphic round nuclei (**Box 31.4**). The nuclei show fine chromatin and inconspicuous nucleoli.[11]

Mucinous Cystic Neoplasia (MCN)[12,13]

Mucinous cystic neoplasia is a very rare lesion in the pancreas and accounts for 2–3% of exocrine pancreatic tumors. This is usually seen in young to middle-aged patients. The tumor may present as small 2–3 cm D cyst to large more than 8 cm cyst. The vast majority of the tumors occur in the body and tail of the pancreas. CT scan of the tumors shows thick-walled multiloculated cystic mass without any connection to the ductal system.

Cytology (Figs 31.3 and 31.4)

Fine needle aspiration cytology of MCN yields thick mucinous material. The smears show abundant mucin along with cohesive cells arranged in monolayered sheets, small tight clusters and

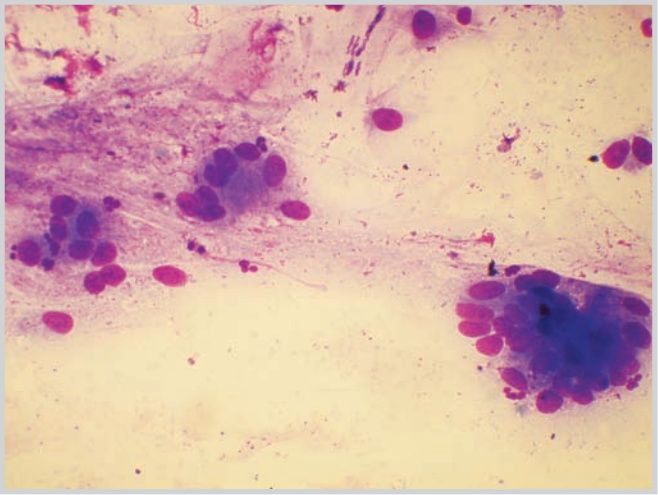

Fig. 31.3: Mucinous cystic neoplasm: Small tight clusters of cells embedded in thick mucinous material (MGG × HP)

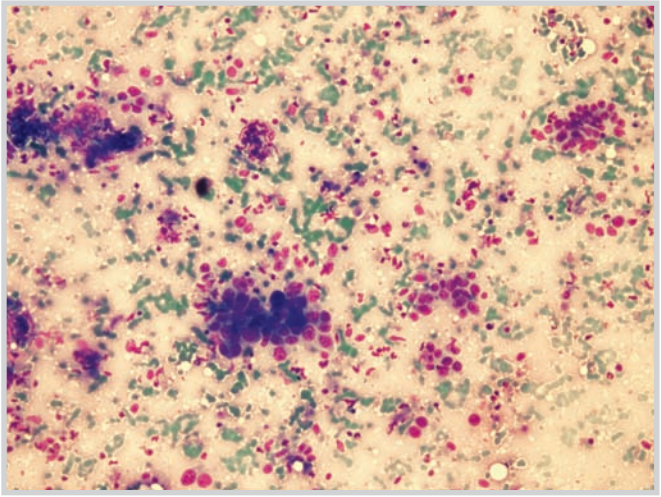

Fig. 31.5: Ductal adenocarcinoma: Multiple loose clusters and discrete malignant cells (MGG × MP)

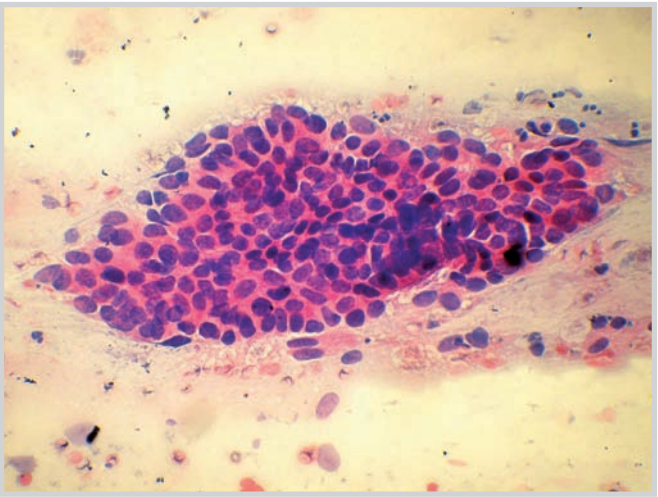

Fig. 31.4: Mucinous cystic neoplasm: Cells with moderate amount of cytoplasm and mildly pleomorphic nuclei (H & E × HP)

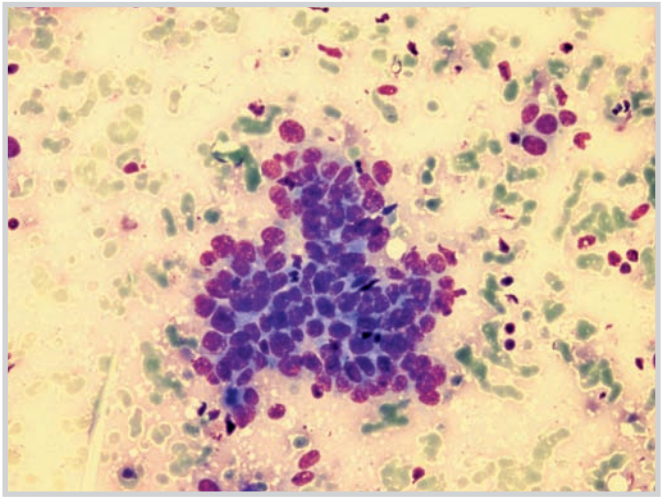

Fig. 31.6: Ductal adenocarcinoma: Cells with nuclear overlapping (MGG × HP)

BOX 31.5 **Mucinous cystic neoplasia**

- Thick mucinous material
- Cells arranged in monolayered sheets, small tight clusters and papillae
- Cell with moderate cytoplasm
- Cytoplasmic vacuolations
- Monomorphic nuclei in benign MCN

papillae-like fashion. Small loosely cohesive cells may be present as freely floating on the mucinous background (**Box 31.5**). The epithelial cells have moderate to abundant vacuolated cytoplasm resembling benign endocervical cells. The nuclei are eccentrically placed with varying degree of atypia. In case of borderline, MCN the nuclei are enlarged and show mild to moderate pleomorphism. Free floating goblet cells may also be seen.

Carcinoma

Ductal Adenocarcinoma[7,8,14-16]

Ductal adenocarcinoma accounts for 85–90% of all pancreatic neoplasm. This tumor exclusively occurs in elderly patient and rarely seen under the age of 40 years. Majority of the carcinomas (60–70%) occur in head of the pancreas. Pancreatic ductal adenocarcinoma is strongly related with cigarette smoking. There is also association of ductal adenocarcinoma of pancreas and intake of diet low in fiber and high in meat. The patient presents with the classical triad of symptoms: jaundice, pain and weight loss.

Cytology (Figs 31.5 to 31.8)

On aspiration cytology smears, the tumor cells are arranged in multiple cohesive clusters, monolayered flat sheets and

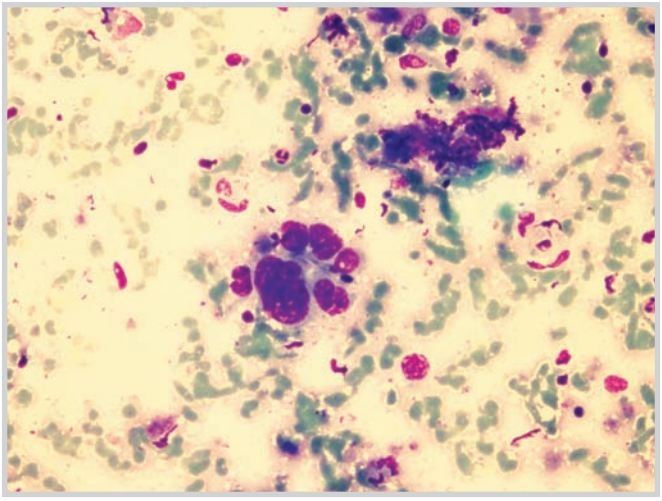

Fig. 31.7: Ductal adenocarcinoma: Nuclear margin irregularity (MGG × HP)

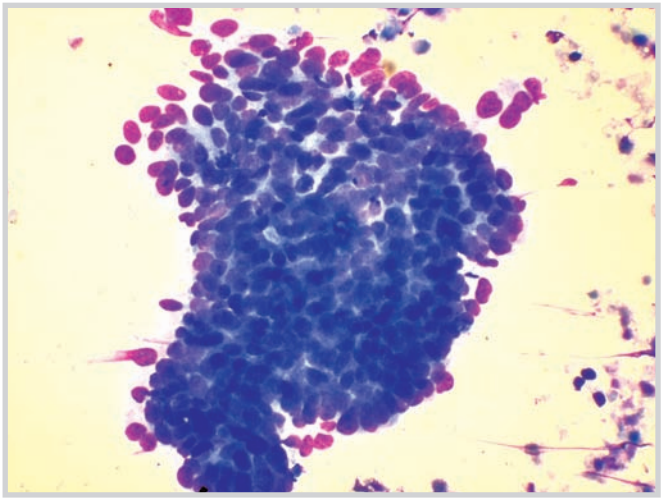

Fig. 31.8: Ductal adenocarcinoma: Large three-dimensional clusters (MGG × HP)

BOX 31.6 Adenocarcinoma of pancreas

- Three-dimensional clusters of cells
- Nuclear overcrowding
- Single discrete cells
- Microacini
- Absence of normal acinar and ductal cells
- Round to oval cells, scanty to moderate cytoplasm
- Anisonucleosis
- Nuclear contour irregularity
- Prominent nucleoli

microglandular pattern. The smears also show many dissociated single cells (**Box 31.6**). The clusters of cells show nuclear overlapping and crowding. Nuclear crowding indicates loss of polarity and this is one of the important diagnostic features

TABLE 31.1: Chronic pancreatitis versus adenocarcinoma of pancreas

Features	*Adenocarcinoma*	*Chronic pancreatitis*
Nuclear crowding and polarity	Nuclear overlapping and loss of polarity	Usually maintained polarity
Microacini	Many	Occasional to absent
Discrete cells	Many	Scanty to absent
Nuclear pleomorphism	Moderate to marked	Mild
Nuclear margin	Irregular	Regular
Nucleoli	Prominent, often large	Inconspicuous

of pancreatic adenocarcinoma. The malignant cells show scanty cytoplasm in nonsecretary carcinomas and moderately vacuolated cytoplasm in secretary (mucinous) carcinoma. The nuclei show moderate pleomorphism with irregular nuclear contour. Nucleoli are prominent. Multivariate logistic-regression analysis of FNAC of pancreatic carcinoma shows three major criteria:[17]

1. Three-dimensional clusters with loss of polarity of the cells
2. Irregular chromatin distribution
3. Nuclear margin irregularity.

Four minor criteria:

1. Nuclear enlargement
2. Single epithelial cells
3. Necrosis, and
4. Mitoses.

Fine needle aspiration cytology diagnosis of moderate and poorly differentiated adenocarcinoma is usually straightforward. It is difficult to diagnose a well differentiated adenocarcinoma of pancreas. Lin F et al. noted that features such as anisonucleosis, nuclear membrane irregularity, nuclear crowding/overlapping/three-dimensional clusters, and nuclear enlargement are the most important criteria to diagnose well-differentiated pancreatic adenocarcinoma.[14]

Differential Diagnosis

- *Chronic pancreatitis:* Cells of chronic pancreatitis may often show nuclear atypia. The cytological features such as nuclear crowding, anisonucleosis, nuclear margin irregularities and altered nucleocytoplasmic ratio favor the diagnosis of adenocarcinoma (**Table 31.1**).
- *Well-differentiated adenocarcinoma:* It is often difficult to distinguish well-differentiated adenocarcinoma from normal pancreatic aspirate. However, nuclear crowding, nuclear margin irregularity, chromatin abnormality and nucleolar prominence favor the diagnosis of well-differentiated adenocarcinoma.

Variants of Ductal Carcinoma

Osteoclastic Giant Cell Carcinoma

This is characterized by multiple multinucleated osteoclast-like giant cells. The nuclei of the giant cells are bland looking.

Anaplastic Carcinoma (Figs 31.9 and 31.10)

Anaplastic carcinoma is also known as giant cell carcinoma or sarcomatoid carcinoma. This tumor represents 2.7% of ductal carcinomas. The prognosis of this tumor is bad. The aspirates show many large bizarre multinucleated giant cells, large pleomorphic epithelial cells and spindle cells. The cells are present in loose clusters or discretely. The background of the smear shows blood, necrosis and inflammatory cells. Small foci of glandular differentiation may also be noted.[18]

Differential Diagnosis

- Malignant melanoma
- Pleomorphic sarcoma

Small Cell Carcinoma

This is an extremely rare carcinoma of pancreas and represents less than 1% of pancreatic malignancies. On cytomorphology, this tumor resembles small cell carcinoma of lung. FNAC smear shows discrete small cells with scanty cytoplasm, small hyperchromatic nuclei and nuclear molding. In fact, before diagnosis of such tumor in pancreas, metastatic small cell carcinoma should be excluded.

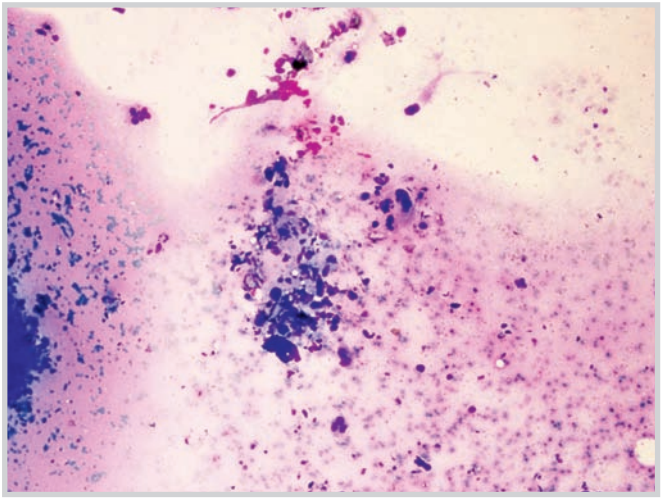

Fig. 31.9: Anaplastic carcinoma: Loose clusters of pleomorphic cells (MGG × MP)

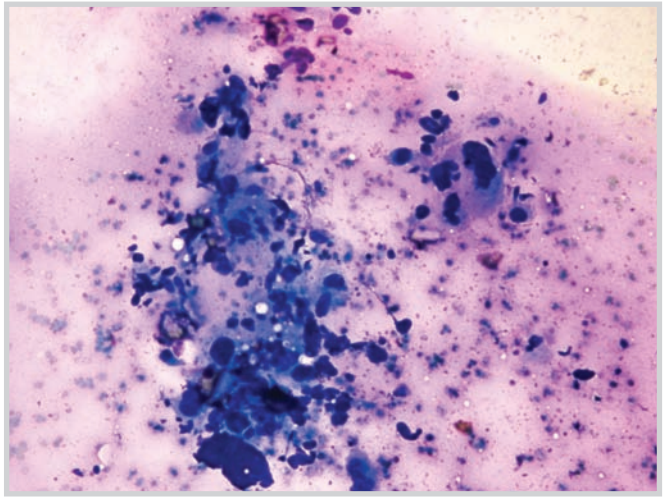

Fig. 31.10: Anaplastic carcinoma: Higher magnification showing markedly enlarged pleomorphic cells (MGG × HP)

Adenosquamous Carcinoma

This is a rare neoplasm and represents only 3–4% of exocrine pancreatic malignancies.[19] It is characterized by the presence of variable proportions glandular elements and squamous components. The cytology smears exhibit dual differentiation with varying proportions of squamous and glandular differentiation. The cell with glandular differentiation shows abundant intracellular mucin with vacuolated cytoplasm and eccentric nuclei. The squamous cells are oval to polyhedral with hyperchromatic nuclei. Tadpole cells are also seen. Intracellular keratinization may be seen in Papanicolaou's staining. The dual differentiation may be focal and overlooked in FNAC smears. In the absence of any squamoid cells the tumor may be diagnosed as poorly differentiated carcinoma.[20]

Signet Ring Carcinoma

This is an extremely uncommon carcinoma of pancreas. Cytology smears show freely floating cells in the pool of mucus. The individual cells have abundant vacuolated cytoplasm with eccentric nuclei. Metastatic signet ring carcinoma from the stomach should be excluded before the diagnosis such tumor.

Pancreatic Acinar Carcinoma[21]

Acinar cell carcinoma of the pancreas is a rare tumor and comprises less than 2% of the exocrine pancreatic neoplasm. This tumor exhibits phenotypic and immunohistochemical evidence of acinar cell differentiation. It is usually seen in adult male. The neoplasm bears a poor prognosis, and 5 years survival rate is less than 10%.[22]

Cytology (Figs 31.11 and 31.12)

Fine needle aspiration cytology smears are richly cellular and the cells show a combination of loose clusters and single cells. Acinar formation is characteristically present and the tumor cells resemble normal pancreatic acinar cells. The cells are large and polygonal with granular cytoplasm (**Box 31.7**). Cytoplasmic granularity is one of the important characteristic features of this tumor. The nuclei are centrally placed mildly pleomorphic round nuclei with smooth nuclear contour. Chromatin is clumped and prominent nucleoli are present.[23]

Differential Diagnosis

- Islet cell tumor
- Solid-pseudopapillary tumors

Neuroendocrine Tumor

Pancreatic Endocrine Tumor (Islet Cell Tumor of Pancreas)[24-26]

The terminology of "islet cell tumor" has been replaced by pancreatic endocrine tumor (PET).[27] It is assumed that the endocrine tumors of pancreas originate from the endocrine

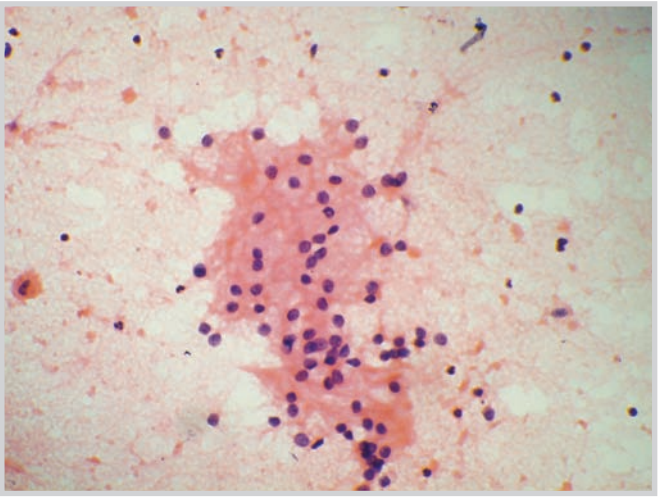

Fig. 31.11: Pancreatic acinar carcinoma: Loose clusters of cells resembling pancreatic acinar cells (H & E × MP)

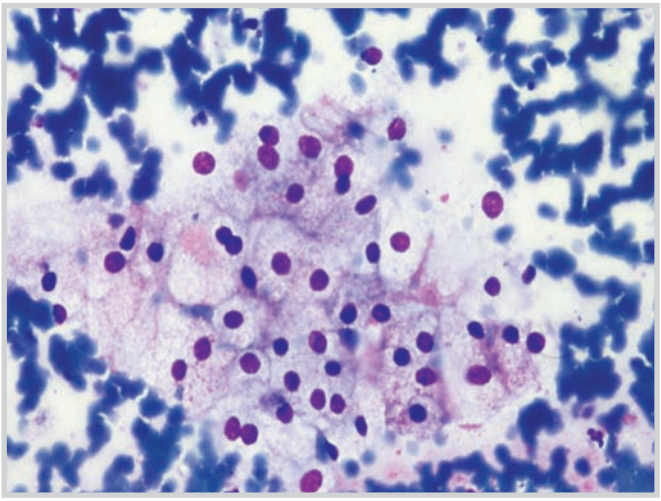

Fig. 31.12: Pancreatic acinar carcinoma: Large polygonal cells with granular cytoplasm having enlarged nuclei (MGG × HP)

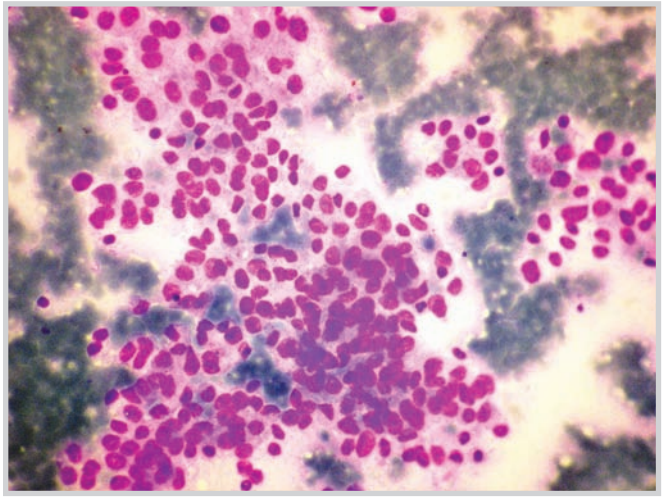

Fig. 31.13: Pancreatic endocrine tumor: Dissociated and loose cohesive clusters of cells (MGG × MP)

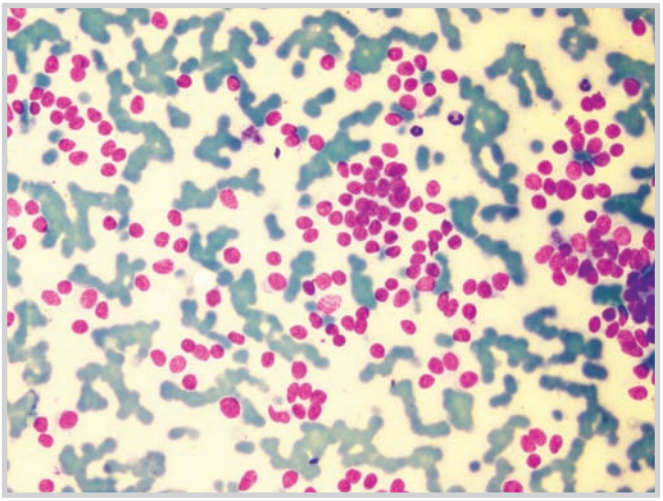

Fig. 31.14: Pancreatic endocrine tumor: Discrete monomorphic cells (MGG × MP)

BOX 31.7 Acinar cell carcinoma

- Loose clusters and discrete cells
- Polygonal cells
- Abundant granular cytoplasm
- Monomorphic round nuclei
- Smooth nuclear contour
- Prominent nucleoli

cells of the diffuse endocrine system. There are about 15 cell types that produce hormonal peptides or biogenic amines in the pancreas. The recognition of specific tumor cell type needs hormone immunohistochemistry. PET is a relatively uncommon tumor and represents less than 5% of all pancreatic tumors. The tumors typically occur in adults; however the children may also be affected by this tumor. PET arises more commonly in body and tail of the pancreas.

The hormonally active or functional PET may secrete various hormones depending on the tumor of the particular type of endocrine cells. The patient may present with severe hypoglycemia in case of insulinoma, peptic ulcer in case of gastrinoma, watery diarrhea in case of vasoactive intestinal polypeptide-producing tumor. Nonfunctioning tumors are asymptomatic and are detected incidentally.

The resection of the tumor cures the low stage tumors and therefore accurate FNAC diagnosis of PET is helpful for the patient.

Cytology (Figs 31.13 to 31.17)

Fine needle aspiration cytology smear shows predominantly dissociated and loose cohesive cells along with trabecular and acinar pattern (**Box 31.8**). The cells are also arranged along the thin-walled capillaries. The individual cells are small to medium sized with scanty to moderate amount of cytoplasm. The cytoplasmic reddish granularity in *May-Grunwald-Giemsa* (MGG) stained smear is one of the important characteristic features of PET.[28] The nuclei are central to eccentric in position,

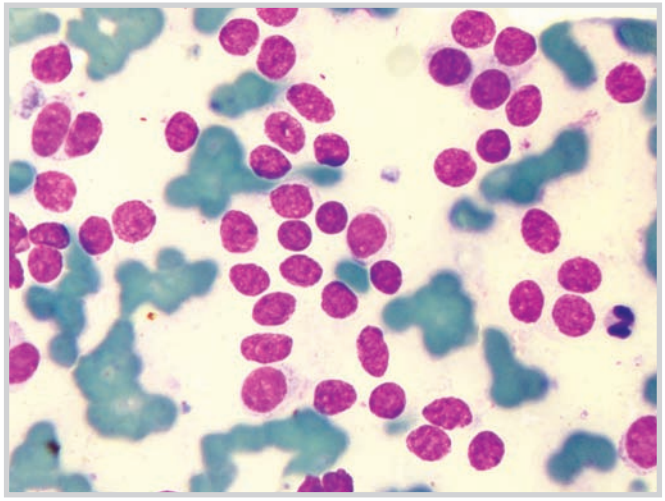

Fig. 31.15: Pancreatic endocrine tumor: Discrete cells with abundant cytoplasm and round monomorphic nuclei having salt and pepper chromatin (MGG × HP)

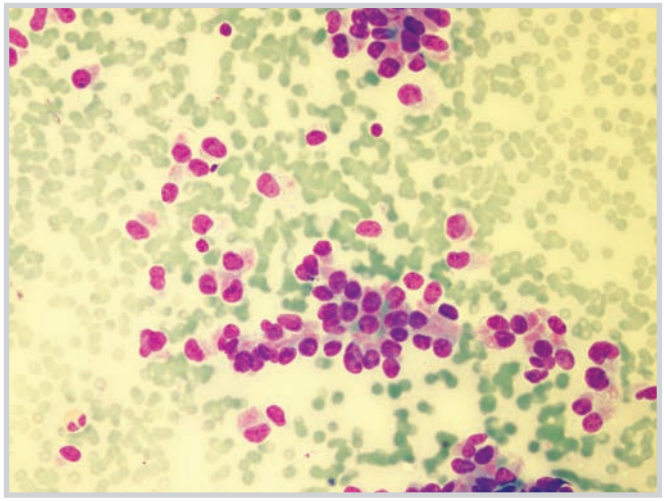

Fig. 31.16: Pancreatic endocrine tumor metastasized in liver: Discrete cells with mild pleomorphism having abundant reddish cytoplasm. No characteristic feature of malignancy is seen (MGG × MP)

round monomorphic and often show bi-nucleation. The nuclear chromatin shows characteristic salt and pepper appearance. The nucleoli are usually inconspicuous. However, occasional prominent nucleoli may be seen. Nuclear pleomorphism and mitotic activity may also be noted and these features are present both in benign and malignant PET. On cytology smear, it is not possible to differentiate benign from malignant PET.

Immunocytochemistry (ICC) stains play an important role in confirmation of the neuroendocrine nature of tumor cells. The most tumors are positive for neuron-specific enolase (NSE), chromogranin A and synaptophysin.

Differential Diagnosis

- *Solid and cystic papillary neoplasm (SCPN):* Cytology smears of SCPN often simulate PET as both the tumors show monomorphic round cells. However, the typical young female, radiological appearance of solid and cystic components and the presence of papillae and macrophages favor the diagnosis of SCPN of pancreas.

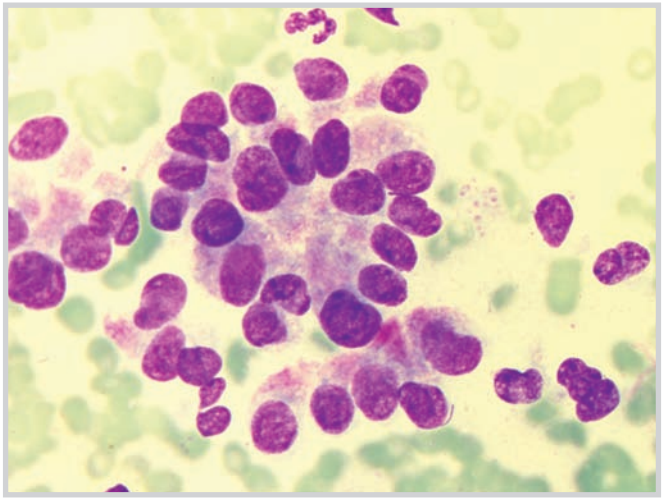

Fig. 31.17: Pancreatic endocrine tumor metastasized in liver: Higher magnification showing better morphology of the cells. Rosette like structure is also seen (MGG × MP)

BOX 31.8 Pancreatic endocrine tumor

- Dissociated cells
- Loose clusters and acinar arrangement
- Round cells with moderate fine reddish granular cytoplasm
- Central to eccentric, monomorphic nuclei
- Salt and pepper chromatin

- *Well-differentiated adenocarcinoma*: Cells in PET are usually discrete and have reddish granular cytoplasm. In contrast, well-differentiated pancreatic adenocarcinoma show cluster of cells with scanty nongranular cytoplasm. Nuclear chromatin of these cells is fine with prominent nucleoli (**Table 31.2**).

Solid and Cystic Papillary Neoplasm of Pancreas[29-31]

This is a relatively uncommon tumor and represents only 1–2% pancreatic tumors. The other name of this tumor is soild-pseudopapillary tumor. This tumor characteristically occurs in young female. USG and CT scan of SCPN show a well circumscribed multilocular cystic mass with both solid and fluid-filled cystic components. The radiologic features are quite suggestive of SCPN in a proper clinical setting.[32] The tumor has low malignant potentiality and most of the patients have a prolonged survival after adequate local surgery.[33]

Cytology (Figs 31.18 to 31.22)

The cytology smears of SCPN show papillae like structures, pesudopapillae, and discrete cells (**Box 31.9**). The branching papillary fronds or pseudopapillae are made of central thin fibrovascular stalks lined by one to more layers of cuboidal tumor cells. The tumor cells are also arranged around the blood vessels. Occasional cases may show acinar or rosettes formation. The discrete cells are monotonous with scanty to moderate cytoplasm and eccentrically placed round nuclei. The cytoplasm is pale or acidophilic and may show vacuolations. The nuclei are monomorphic with fine chromatin having occasional small

TABLE 31.2: Pancreatic endocrine tumor versus well differentiated adenocarcinoma

Pancreatic endocrine tumor	*Pancreatic well-differentiated adenocarcinoma*
Discrete cells	Clusters and glandular
Moderate amount of reddish granular cytoplasm	Scanty amount of vacuolated cytoplasm
Regular, monomorphic nuclei with salt and pepper chromatin	Irregular and hyperchromatic nuclei with prominent nucleoli
Positive for chromogranin and synaptophysin	Negative for chromogranin and synaptophysin

BOX 31.9 **Solid and cystic papillary neoplasm**

- Papillae with fibrovascular core
- Small round cells
- Scanty to moderate cytoplasm
- Eccentric nucleus
- Fine chromatin
- Nuclear grooves
- Foamy macrophages
- Positive for CD10, NSE and CK

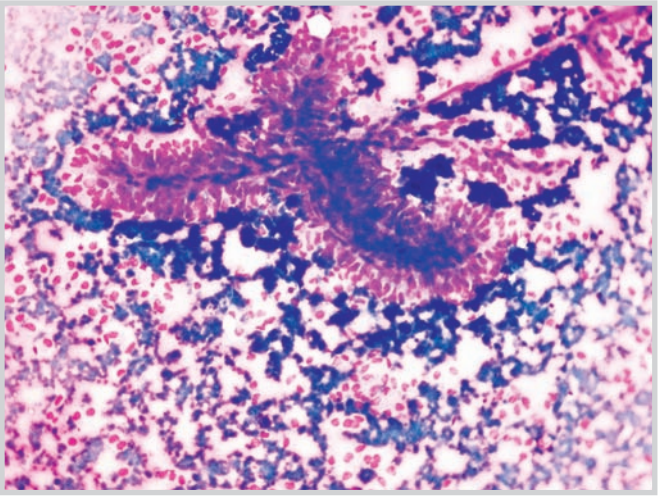

Fig. 31.18: Solid and cystic papillary neoplasm of pancreas: Papillary structure and discrete cells (MGG × MP)

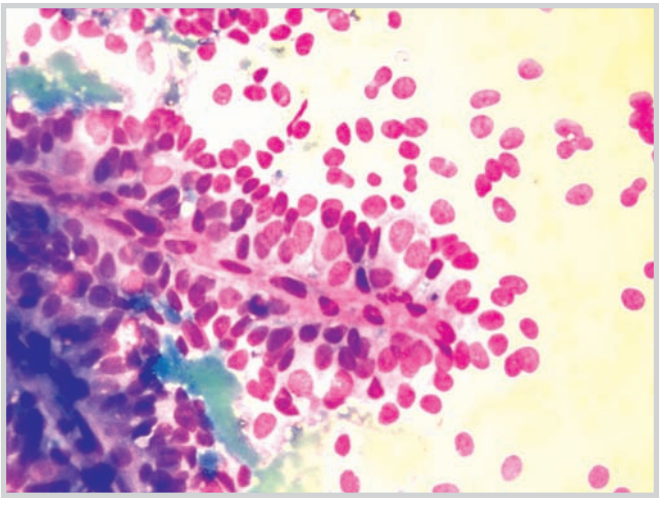

Fig. 31.19: Solid and cystic papillary neoplasm of pancreas: Higher magnification of the papillary structure (MGG × HP)

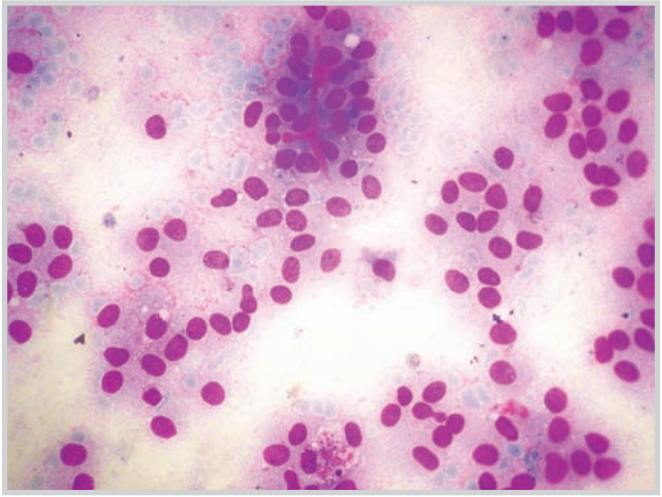

Fig. 31.20: Solid and cystic papillary neoplasm of pancreas: Discrete round monomorphic cells with reddish moderate amount of cytoplasm (MGG × HP)

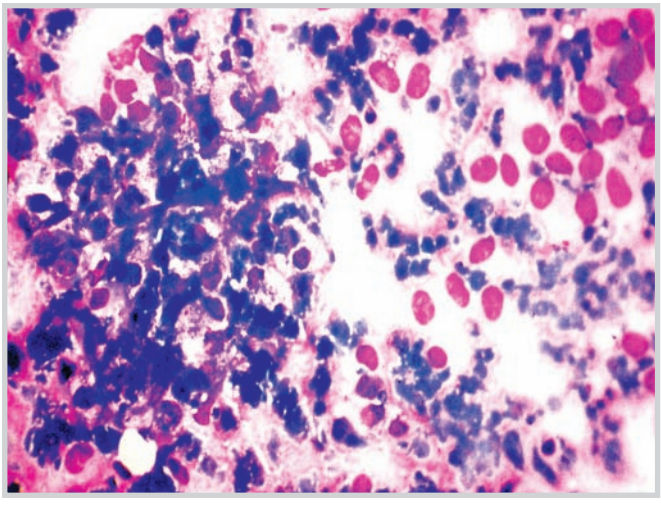

Fig. 31.21: Solid and cystic papillary neoplasm of pancreas: Clusters of foamy macrophages are seen (MGG × MP)

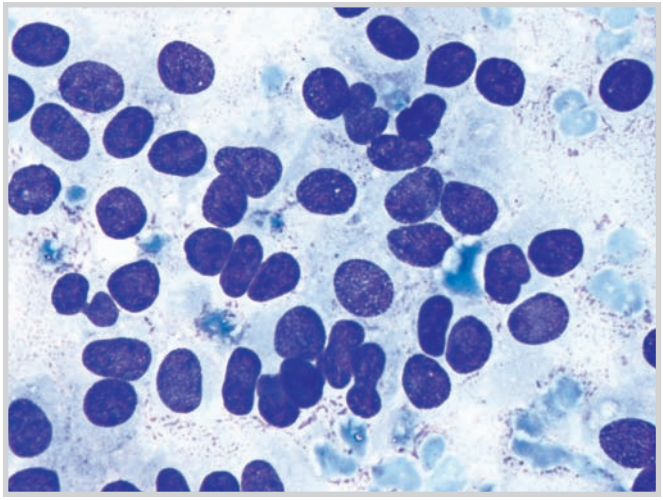

Fig. 31.22: Solid and cystic papillary neoplasm of pancreas: Cell shows round nuclei with stippled chromatin and inconspicuous nucleoli [May-Grünwald-Giemsa (MGG) stain × oil immersion (OI)]

nucleoli. Nuclear pleomorphism, indentations or prominent infoldings, and grooves of the nuclear membrane have been occasionally described.[34] Degenerative changes such as hyaline droplets or pink cytoplasmic inclusions in MGG stain have also been described. Many foamy macrophages are also noted in the background.

Immunohistochemistry: Solid and cystic papillary neoplasm shows keratin, vimentin, desmoplakin, trypsin, chymotrypsin, amylase, galectin-3, CD10, and CD56 positivity. The cells also show positivity for NSE and other neuroendocrine markers.

Differential Diagnosis

- *Islet cell tumors:* see above
- *Papillary carcinoma:* The presence of multiple papillary structures may be confused with that of papillary ductal carcinoma of pancreas. The discrete monomorphic cell component is helpful distinguishing feature.

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CHAPTER 32

Kidney and Adrenal

Chapter Contents

INTRODUCTION

There are many diagnostic modalities of the space occupying lesions of the kidney such as ultrasonography (USG), computed tomography (CT) scan and magnetic resonance imaging (MRI). The diagnostic accuracy of these imaging technologies is quite high. These investigative procedures have considerably reduced the need of fine needle aspiration cytology (FNAC). However, FNAC is still indicated:

- To confirm the diagnosis of kidney lesions, particularly where radiological diagnosis is equivocal
- In advanced cases of renal cell carcinoma where radical surgery is not needed, to confirm metastasis from the other lesions
- Radical nephrectomy is contraindicated due to medical reason
- Small kidney lesions in young patient who needs partial nephrectomy
- Therapeutic application of cyst fluid evacuation
- Suspected cases of pyelonephritis where the patient can be treated medically.

Fine needle aspiration cytology of the kidney lesion is usually done by USG, CT or MRI guidance. USG guided FNAC is easy, cheap and flexible, whereas CT scan can localize small lesion with better resolution. FNAC is done with a 22 gauze needle and stylet. The patient is kept in prone position and local anesthesia is applied at the site of FNAC. Both air-dried and alcohol-fixed smears are made. It is always preferable to take sample for cell block for immunostaining. Complications of FNAC of the kidney are rare. Local hemorrhage, pain, hematuria and pneumothorax are occasionally encountered. Needle tract seeding due to FNAC is extremely rare and does not occur if thin bore needle is used.[1] The diagnostic sensitivity of FNAC is more than 80% with a high specificity rate of near about 98%.[2-5] False negative cases are mainly due to sampling error. Well-differentiated renal cell carcinoma (RCC) may be mistaken as benign cells. False positive reports are rare. This may occur particularly in cases of angiomyolipoma (AML) and xanthogranulomatous pyelonephritis.

NORMAL CELLS

Fine needle aspiration cytology of normal kidney elements are predominantly tubular cells which are derived from the distal convoluted tubules, proximal convoluted tubules and the loop of Henle.

- *Tubular cells* **(Fig. 32.1):** Tubular cells are usually present as small clusters and discretely. They have abundant granular cytoplasm with indistinct border. The nuclei are monomorphic round, and central in position. Chromatin of the nuclei are fine granular with inconspicuous nucleoli. The cells often contain coarse dark lipofuscin pigments.

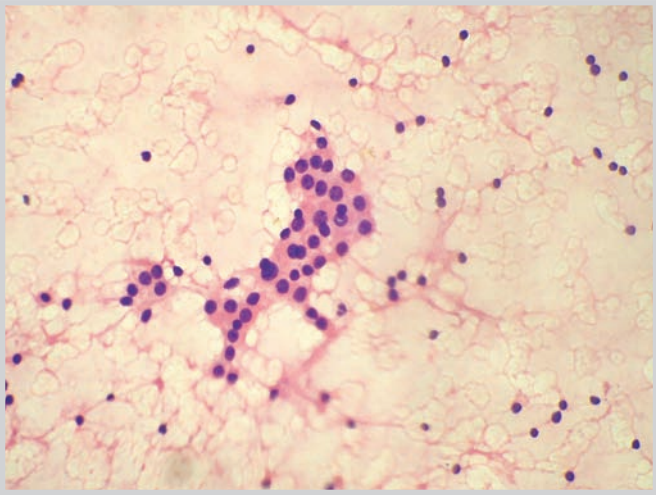

Fig. 32.1: Renal tubular cells: small clusters and discrete cells with abundant indistinct cytoplasm and monomorphic round nuclei [hematoxylin and eosin (H & E) × medium power (MP)]

- *Endothelial cells*: Oval to spindle-shaped endothelial cells may also be noted in the background.
- *Glomerular fragments*: Uncommonly fragments of glomerulus may be aspirated. These are cohesive clusters of small round cells with scanty cytoplasm. These cells are surrounded by the thin capillaries.

RENAL LESIONS

Renal Cyst[6]

Renal cysts are very common and represent near about 70%–80% of all renal lesions.[7] Most of these cysts are benign lesion and rarely may represent cystic RCC. The majority of the benign renal cysts are accidentally detected in the course of investigation of other diseases. The cysts are diagnosed accurately by USG and rarely needs cytological examination. However, in selected conditions, the cyst should be distinguished from the cystic renal tumor. Bosniak MA has classified renal cyst in four categories based on radiological imaging:[8,9]

- Category I: Clearly simple cysts.
- Category II: These cysts are a little more complicated and contain some thin smooth septa, minimal wall thickening and minimal calcification. These cysts are clearly not worrisome.
- Category III: These cysts are more complicated with irregular thick septa or wall. They may be the feature of both benign and malignant lesions.
- Category IV: These are clearly malignant lesions and show enhanced soft tissue areas adjacent to or separate from the wall.

The cysts of category I are all benign and do not need any FNAC. Whereas, the cysts of category IV type needs surgical intervention.

Cytology

Evacuation of the large simple cyst is therapeutically helpful. FNAC of the benign renal cyst usually yields thin clear fluid

BOX 32.1 Renal cyst

- Clear fluid
- Foamy macrophages
- Degenerated epithelial cells

(**Box 32.1**). The aspirate shows scanty cellularity. There may be scattered foamy macrophages along with bland looking renal epithelial cells. In occasional cases, the tubular epithelial cells may show mild degree of nuclear atypia. Atypia in the category II and III renal cyst should be considered as significant.

Differential Diagnosis

Renal cell carcinoma: Occasionally, RCC may show considerable cystic changes and may be difficult to distinguish from a simple cystic lesion. Nuclear atypia in RCC may be helpful in this aspect.

Xanthogranulomatous Inflammation

Xanthogranulomatous inflammation is a chronic inflammatory condition characterized by recurrent urinary tract infection, flank pain and solid space occupying lesion in kidney. The lesion may mimic RCC on radiology.

Cytology (Figs 32.2 and 32.3)

The cytology smear shows large clusters of foamy histiocytes around the capillaries. Multinucleated giant cells may also be seen. The individual cells have abundant vacuolated cytoplasm with central round bland nuclei. Unlike RCC, the nuclei of the histiocytes do not show any atypia. Necrosis and lymphocytes may be seen in the background.

Differential Diagnosis

Renal cell carcinoma.

Angiomyolipoma[8,9]

Angiomyolipoma is an uncommon benign renal tumor composed of intimate admixture of adipose tissue, smooth muscle and blood vessels. It represents about 1% of all surgically resected renal mass. Almost 50% of AML occurs with tuberous sclerosis, an autosomal inherited disorder.[10] An USG examination and angiography may suggest a diagnosis of AML, particularly due to high fat content. However, FNAC is indicated when the lesion contains low amount of fat and cannot be distinguished from RCC. The patients without tuberous sclerosis presents with flank pain, hematuria and solid renal mass. However, the patients with tuberous sclerosis are asymptomatic. The correct preoperative diagnosis of AML is necessary for the conservative surgery and therefore FNAC has a definitive role in such cases. The salient diagnostic features of AML are mentioned in **Box 32.2**.

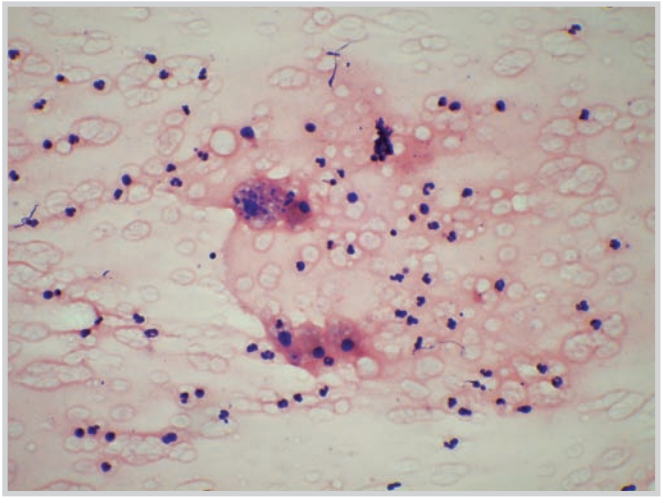

Fig. 32.2: Xanthogranulomatous inflammation: discrete foamy histiocytes and lymphocytes (H & E × MP)

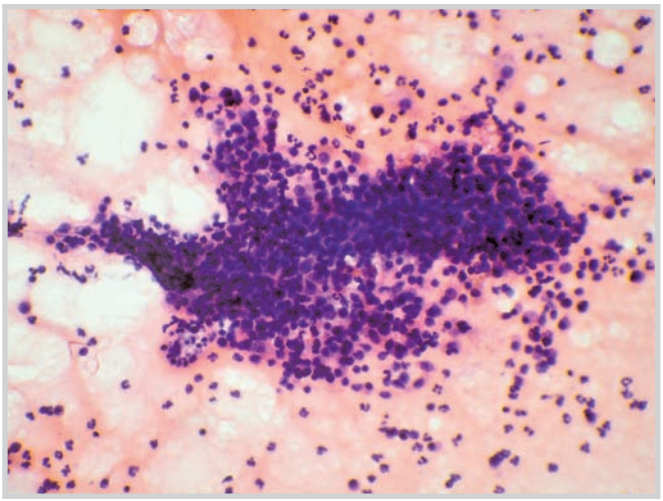

Fig. 32.3: Xanthogranulomatous inflammation: renal tubular calls admixed with foamy histiocytes and lymphocytes (H & E × MP)

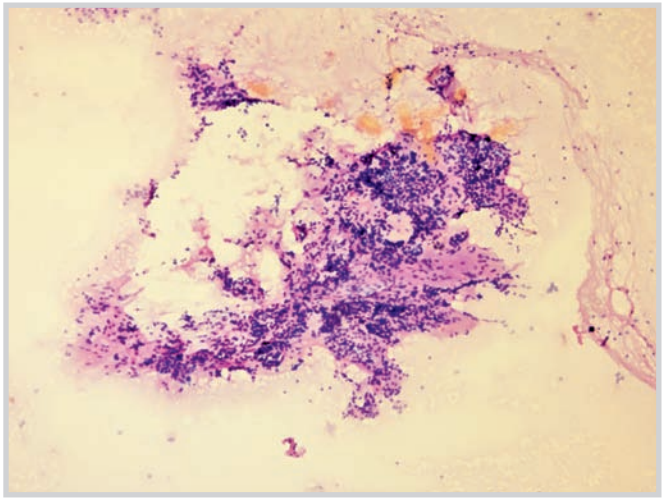

Fig. 32.4A: Angiomyolipoma: clusters and discrete oval to spindle cells along with renal tubular cells [H & E × low power (LP)]

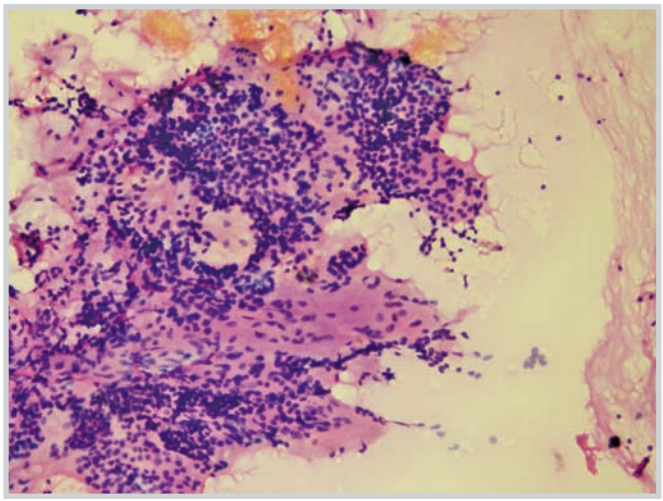

Fig. 32.4B: Angiomyolipoma: spindle cells embedded in the stroma [H & E × high power (HP)]

BOX 32.2 Angiomyolipoma

- Mature fat cells
- Thick-walled blood vessels
- Round to spindle-shaped mesenchymal cells
- Vacuolated cytoplasm
- Epithelioid cells with nuclear atypia
- Computed tomography scan characteristics

Cytology (Figs 32.4A and B, Fig. 32.5)

Smear shows abundant clusters of mature fat cells. There are multiple fragments of thick-walled blood vessel fragments. The vessels are lined by endothelial cells. The leiomyomatous cells are present in small clusters or in dissociation. These cells are round to spindle-shaped with moderate amount of vacuolated cytoplasm.[11] The nuclei are oval to spindle-shaped with blunt ends giving rise to cigar shaped appearance. The nuclear chromatin is fine granular with single prominent nucleoli. Occasionally, these smooth muscle cells with spindle-shaped nuclei show marked nuclear pleomorphism and these cells should be carefully interpreted to avoid false positive diagnosis of sarcomatoid RCC.

The cells of AML are positive for HMB-45 and Melan A. These are useful markers to differentiate AML from RCC.

Differential Diagnosis

Renal cell carcinoma: The presence of atypical mesenchymal cells with bizarre nuclei may mislead the cytologist to the diagnosis of RCC. However, the nuclear chromatin of the cells of AML is bland and the nucleoli are inconspicuous. The presence

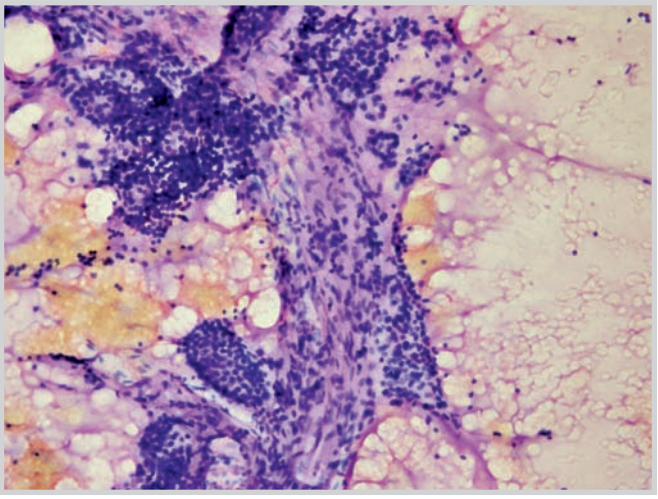

Fig. 32.5: Angiomyolipoma: spindle cells are arranged in long bundles (H & E × HP)

of adipose tissue usually favors AML. CT scan features are usually characteristic of this tumor.

Renal Cell Carcinoma[2,4,5,12-14]

Renal cell carcinoma arises from the renal tubules and is the most common primary malignant tumor in the kidney. RCC represents about 90% of the malignant tumors of the kidney. It generally occurs in the adults of late fifties and males are commonly affected than female. The children may also develop RCC. Tobacco smoking, obesity and exposure to carcinogenic arsenic compounds are the common risk factors of RCC. A small proportion (4%) of RCC occurs as hereditary cancer.[15] Von Hippel-Lindau (VHL) tumor suppressor gene is located on chromosome 3p25. Inactivation of this tumor suppressor gene plays an important role in hereditary and sporadic clear cell renal carcinoma. Based on molecular cytogenetics and histogenetic study of RCC, it is strongly recommended that these tumors are histogenetically heterogeneous group of tumors with significantly different prognostic outcome.

Classification based on molecular cytogenetics and histology:[16,17]

- Conventional (clear cell) renal carcinoma:
  - Seventy percent cases
  - Proximal convoluted tubules in origin
  - del3p
- Papillary renal carcinoma:
  - Ten to fifteen percent cases
  - Distal convoluted tubules in origin
  - Trisomies of chromosomes 3q, 7, 12, 16, 17 and 20
  - Prognostically good
- Chromophobe renal carcinoma
  - Five percent cases
  - Intercalated cells origin
  - Monosomy of multiple chromosomes (1, 2, 6, 10, 13, 17 and 21) and hypodiploidy
- Collecting duct carcinoma:
  - Less than 1%
  - Collecting duct origin
  - No consistent cytogenetic abnormality
- Renal cell carcinoma, unclassified
  - Four to five percent
  - Do not fit into any category

The patients of RCC usually present with the classical triad of hematuria, flank pain and abdominal mass. However, 40% patients of RCC may present with loss of weight loss, abdominal pain, anorexia and fever.

Cytology (Figs 32.6 to 32.8)

Clear Cell Type of Renal Cell Carcinoma

Near about 75% of RCC is clear cell carcinoma. The FNAC smear of clear cell carcinoma is usually highly cellular (**Box 32.3**). The cells are arranged in large cohesive clusters and singly. Transgressing blood vessels within the clusters of cells are also noted. The individual cells have abundant cytoplasm with distinct cytoplasmic outline. Nucleocytoplasmic ratio of the cell is low. The nuclei are round and variably enlarged. The nuclear position is central to eccentric. Depending on the grade of the tumor, the cells show nuclear enlargement and nucleolar prominence. The higher grade tumors have moderate to markedly enlarged nuclei with irregular nuclear contour. There is frequent binucleation. Nuclear chromatin is fine to coarse depending on the grading of the tumor. Nucleoli may be indistinct to large and prominent. It is important to differentiate the benign tubular cells and low-grade renal cell carcinoma as they may often simulate each other (**Table 32.1**).

Papillary Carcinoma

The papillary renal carcinoma is defined as a malignant tumor of the kidney composed of more than 50% papillae. Smears show multiple papillae and cohesive three-dimensional clusters of cells (**Box 32.4**). On cell block, the papillae may show fibrovascular core. The individual cells show dense cytoplasm and small round relatively monomorphic bland nuclei. The nucleoli are inconspicuous to absent. The nuclei often show deep nuclear grooves and intranuclear pseudoinclusion. Occasional cases show intracytoplasmic hemosiderin pigments. In minority of the cases, psammoma bodies are also seen. Higher grade papillary carcinoma shows cells with abundant granular cytoplasm having enlarged nuclei and large nucleoli. The higher grade papillary carcinoma is difficult to distinguish from the clear cell carcinoma.

Differential Diagnosis

Clear Cell Renal Cell Carcinoma

The presence of occasional papillae in the ordinary RCC may be confused with papillary carcinoma. The relatively bland nuclei, nuclear grooving, intracellular hemosiderin and the presence of psammoma bodies favor the diagnosis of papillary carcinoma.

***Chromophobe Type*:** Chromophobe RCC comprises of 5% of all RCCs. The FNAC smears are highly cellular and consist of small groups and isolated cells. The individual cells show abundant floppy, granular cytoplasm with well-defined cytoplasmic margins (**Box 32.5**). In May-Grünwald-Giemsa (MGG) stained smears, the cytoplasm shows perinuclear reticulated zone instead of typical clear vacuolated area.

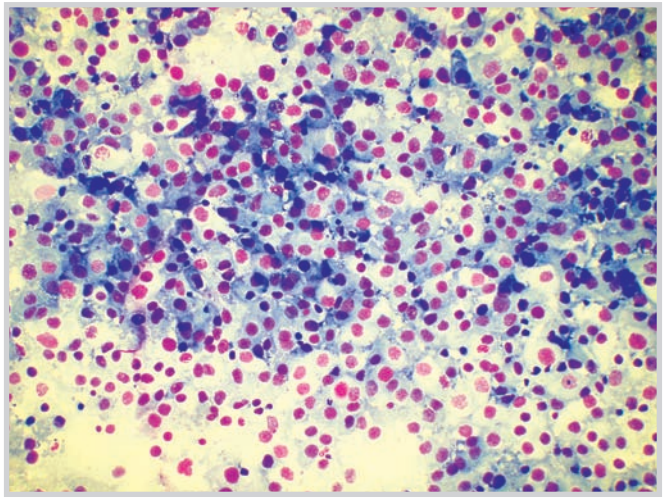

Fig. 32.6: Renal cell carcinoma: abundant discrete cells with clear cytoplasm and mildly enlarged nuclei in a well-differentiated renal cell carcinoma [May-Grünwald-Giemsa (MGG) × MP)]

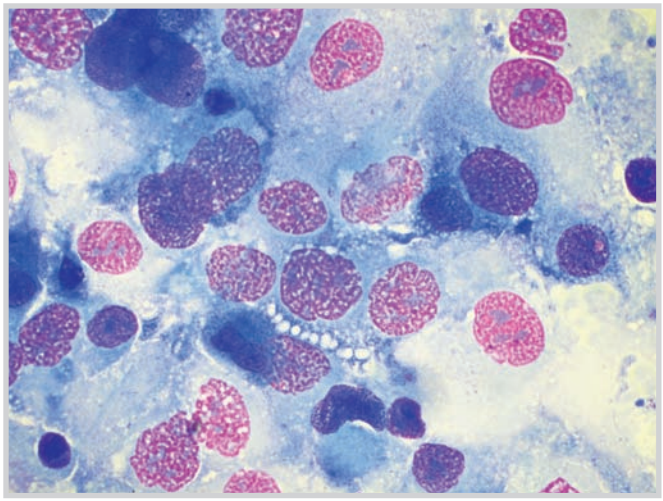

Fig. 32.8: Renal cell carcinoma: higher magnification showing better morphology of the cells. The cells have abundant cytoplasm. Nuclei show fine chromatin and prominent nucleoli [MGG × oil immersion (OI)]

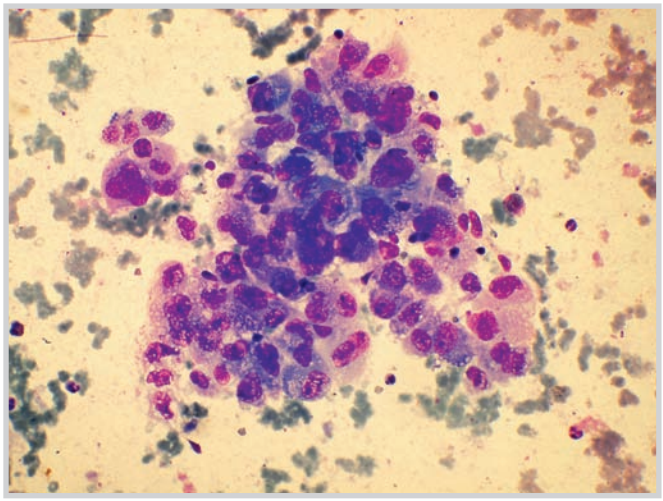

Fig. 32.7: Renal cell carcinoma: loosely cohesive clusters of cells with moderate amount of vacuolated cytoplasm. Nuclei are moderately enlarged and pleomorphic (MGG × HP)

TABLE 32.1: Differentiating features between clear cell carcinoma and benign tubular cells

Features	*RCC cells*	*Benign tubular cells*
Cells	Large cohesive clusters	Discrete
Cytoplasm	Vacuolated	No vacuolations
Nuclei	Enlarged: mild to moderate	No enlargement
Nucleoli	Prominent	Inconspicuous to absent

Abbreviation: RCC, renal cell carcinoma

BOX 32.3 Renal cell carcinoma

- Multiple clusters, papillary fragments and discrete cells
- Cell with well-defined cytoplasmic outline
- Abundant granular or finely vacuolated pale cytoplasm
- Central round nuclei with variable pleomorphism
- Fine to coarse nuclear chromatin depending on grade
- Frequent binucleation
- Inconspicuous to large prominent nucleoli
- Immunocytochemistry: Positive for cytokeratin, vimentin and CD10.

BOX 32.4 Papillary carcinoma of kidney

- Multiple papillae and cohesive clusters of cells
- Small round cells
- Monomorphic bland nuclei
- Deep nuclear groove
- Intranuclear pesudoinclusion
- Intracytoplasmic hemosiderin pigments
- Psammoma bodies

BOX 32.5 Chromophobe renal cell carcinoma (RCC)

- Abundant floppy, granular cytoplasm
- Well-defined cytoplasmic margins
- Perinuclear reticulated zone
- Intranuclear pesudoinclusion
- Round moderately pleomorphic nuclei
- Frequent binucleation
- Intranuclear pesudoinclusion

Cytoplasmic vacuolations are usually not seen. The nuclei are round, moderately pleomorphic and show frequent binucleation. Nuclear margin may be irregular and intranuclear pesudoinclusion may be seen.[18] Chromophobe cell carcinoma may often simulate as oncocytoma as both the tumor shows cells with abundant granular cytoplasm and frequent binucleation. However, nuclear pleomorphism is more severe in chromophobe cell carcinoma. Chromophobe RCC may also mimic clear cell RCC on cytology. The cells of chromophobe RCC are dispersed, and more pleomorphic than clear cell RCC. Chromophobe RCC is positive for cytokeratin-7 (CK7) and C-Kit. The cells are negative for vimentin.

***Sarcomatoid Type*:** This tumor comprises of only 1% of RCC. It is relatively aggressive in behavior. The tumors usually have the admixture of sarcomatoid and epithelial components. FNAC smears show predominantly clusters and dissociated cells spindle cells in a necrotic or clean background. The cells have moderate to marked pleomorphic spindle-shaped nuclei with irregular nuclear margin and large prominent nucleoli. These cells are admixed with high-grade epithelial component with abundant clear or granular cytoplasm. Osteoid, cartilaginous fragments and multinucleated giant cells may also be seen. In case of lack of epithelial component, CK positivity is required to confirm the diagnosis.

Angiomyolipoma

The presence of only spindle cells and complete lack of epithelial component may simulate AML. However, the spindle cells are less pleomorphic and there may be other components such as fat in the smear of AML.

Collecting Duct Type

This is a very rare type of RCC. The tumor probably arises from the collecting ducts. The FNAC smear shows single and clusters of cells. Occasional papillary arrangements of cells are also noted. The cells are relatively small with scanty to moderate cytoplasm and round hyperchromatic nuclei. Psammoma bodies are also noted.[19] The tumor may often be mistaken as metastatic carcinoma.

Nuclear grading[12,13] of RCC has been discussed in **Table 32.2**. **Table 32.3** describes the cytological features of different types of RCC.

- Benign renal tubular cells
- Hepatocytes: At times, hepatocytes with abundant granular cytoplasm with reactive atypia may be confused with the cells of low-grade RCC. The hepatocytes are polygonal in shape with centrally placed nuclei having very low nucleocytoplasmic ratio. The cytoplasm of the hepatocyte often contains bile pigments.
- Angiomyolipoma
- Xanthogranulomatous pyelonephritis: Discussed in xanthogranulomatous pyelonephritis section.
- Benign cysts
- Adrenal tumor: At times, it is very difficult to differentiate a clear cell RCC located in the upper pole of kidney from an adrenocortical carcinoma. **Table 32.4** describes the possible differentiating points to distinguish adrenocortical carcinoma from RCC.

TABLE 32.2: Nuclear grading of renal cell carcinoma

Grade	*Nuclear size*	*Nucleoli*
Grade 1	Small, round regular	Inconspicuous to absent
Grade 2	Mildly enlarged, irregular	Small conspicuous
Grade 3	Moderately enlarged, irregular	Prominent
Grade 4	Severely pleomorphic enlarged	Macronucleoli

Oncocytoma

Oncocytoma is a benign renal epithelial neoplasm that comprises of less than 3% of all renal tumors. The majority of the patients are asymptomatic at the time of diagnosis.

Cytology (Figs 32.9 and 32.10)

The FNAC smear shows predominantly dissociated cells. The cells are rarely arranged in clusters (**Fig. 32.6**). The individual cells have abundant eosinophilic granular cytoplasm with well-defined cell border. Cytoplasm of the cells does not show any vacuolations. The nuclei are bland, round and monomorphic with inconspicuous nucleoli. Oncocytoma cells are negative for Hale's colloidal iron staining.

Diagnostic Difficulties

- *Chromophobe type of renal cell carcinoma*: Oncocytoma can be confused with chromophobe type of RCC. These cells are positive for Hale's colloidal iron staining.
- *Hepatocytes*: Normal hepatocytes have also abundant granular cytoplasm and these cells may be aspirated in case of FNAC of right kidney. Hepatocytes may rarely be mistaken as oncocytes.

Urothelial Carcinoma

Urothelial carcinoma (UC) was previously known as transitional cell carcinoma. It represents about 7% of all primary carcinomas of kidney. The tumor is often multifocal in origin. The common risk factors of the UC are cigarette smoking, analgesic abuse, renal papillary necrosis and renal stone. The patient usually presents with painless, hematuria, flank pain and palpable mass.

Cytology (Figs 32.11 and 32.12)

Smear shows usually small clusters, papillae and single cells. The individual cells are elongated, spindle-shaped with glassy homogenous cytoplasm (**Box 32.7**). The nucleocytoplasmic ratio

TABLE 32.3: Cytological features of different types of renal cell carcinoma

Type	*Cell pattern*	*Cytoplasm*	*Nucleus*	*Additional features*	*Immunocytochemistry*
Clear	Clusters	Abundant, clear vacuolated	Round, low N/C ratio, monomorphic		Negative for CK7, C-Kit and positive for EMA and CD10
Papillary	Papillae	Moderate dense	Round, low N/C ratio, monomorphic	Necrosis present, intranuclear inclusions, psammoma bodies	Positive for CK7,EMA, Vimentin and CD10
Sarcomatoid	Dissociated	Mild to moderate	Spindle, shaped, high N/C ratio, pleomorphic	Necrosis present, malignant epithelial component	Positive for CK and vimentin
Chromophobe	Dissociated	Abundant floppy, granular	Round, low N/C ratio, monomorphic		Positive for C-Kit

Abbreviations: N/C, nucleus-to-cytoplasm; EMA, epithelial membrane antigen; CK, cytokeratin

TABLE 32.4: The clinical, cytological, immunocytochemistry and electron microscopy to distinguish adrenocortical carcinoma from renal cell carcinoma

	Renal cell carcinoma	*Adrenocortical carcinoma*
Cushing's syndrome	Absent	May be present
Cell arrangement	Papillary and tubular	Discrete and clusters
Cytoplasmic glycogen	Present	Usually absent
Immunocytochemistry: Keratin AE1 and AE3 EMA Vimentin Mela A Inhibin alpha Calretinin	 Positive Positive Positive Negative Negative Negative	 Negative Negative Positive Positive Positive Positive
Electron microscopy	Microvillus present	Mitochondria with tubular or vesicular cristae

Abbreviation: EMA, epithelial membrane antigen

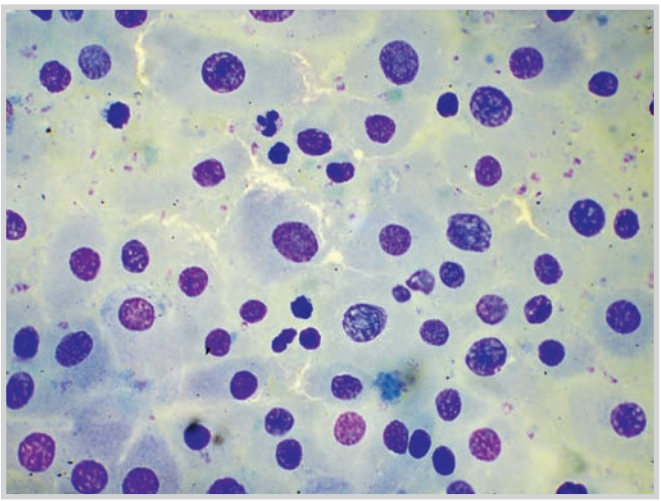

Fig. 32.9: Renal onocytoma: discrete cells with abundant cytoplasm and monomorphic round nuclei (MGG × HP)

is low to medium. Depending of the grade of the tumor, the nuclei show mild to moderate pleomorphism. In many cases, elongated cells with thin long tail of cytoplasm, known as cercariform cells, are seen. These cells have wide cytoplasm in the ends and thin in the middle.[20]

Differential Diagnosis

- *Papillary renal cell carcinoma*: The papillary RCC shows histiocytes and occasionally psammoma bodies.
- *Reactive transitional cells*: Abundant reactive transitional cells are seen in renal stone and a false positive diagnosis of papillary transitional cell carcinoma (TCC) may be given. Nuclear morphology is helpful in differentiating TCC from reactive transitional cells.

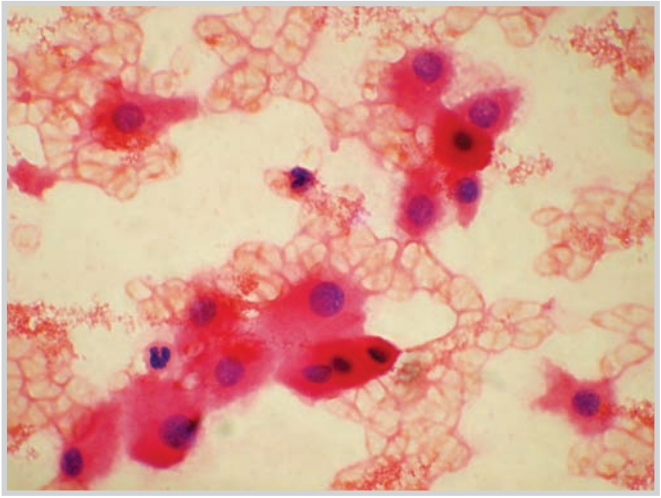

Fig. 32.10: Renal onocytoma: cells with well-defined cytoplasmic border and eosinophilic cytoplasm (H & E × OI)

BOX 32.6 **Oncocytoma**

- Dissociated cells
- Abundant granular eosinophilic cytoplasm
- Small bland nuclei

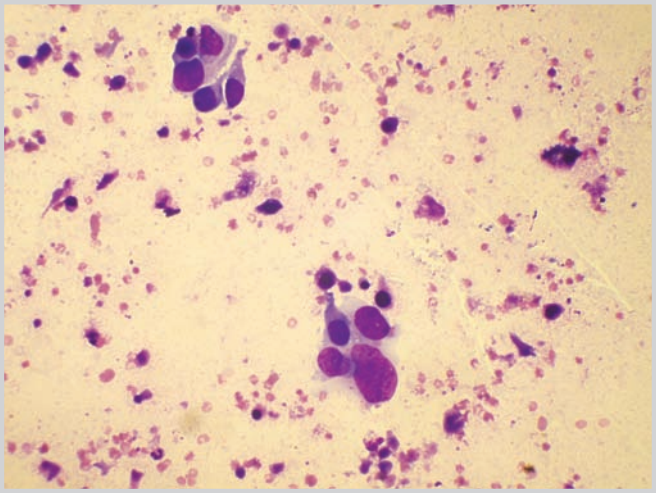

Fig. 32.11: Urothelial carcinoma: oval to elongated cells with basally placed nuclei (MGG × HP)

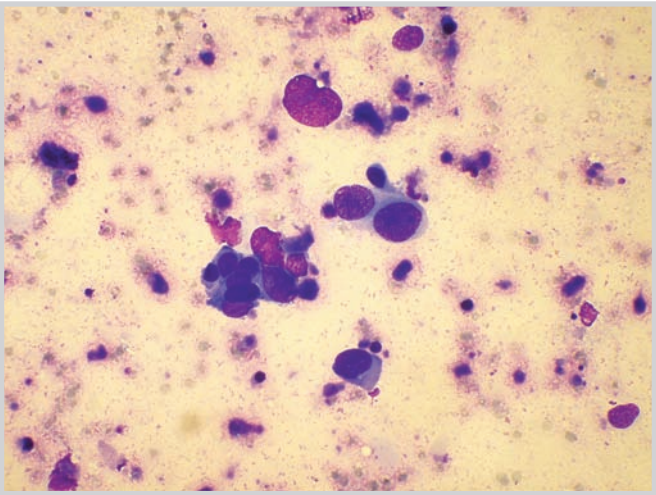

Fig. 32.12: Urothelial carcinoma: columnar looking cells with moderately pleomorphic nuclei (MGG × HP)

BOX 32.7 **Urothelial carcinoma**

- Clusters and papillae
- Elongated cells
- Medium to low nucleocytoplasmic ratio
- Glassy homogenous cytoplasm
- Cercariform cells

Metastatic Tumors of Kidney

Metastasis in kidney represents only 7% of renal tumor. Metastatic kidney rarely presents any symptomatic manifestation. Lung is the commonest primary site followed by breast, colon and pancreas. It is very difficult to recognize the metastatic tumor on FNAC smear. However, if the tumor affects bilateral kidneys with a history of primary tumor and the smear shows large pleomorphic cells or small undifferentiated cells then a possibility of metastatic tumor should be considered.

PEDIATRIC RENAL TUMORS

Nephroblastoma (Wilms' Tumor)[21,22]

Nephroblastoma is also known as Wilms' tumor (WT). It is primarily a neoplasm of the infants that develops from the nephrogenic blastemal cells. The tumor occurs in both sexes with equal frequencies and 98% of the tumor occurs before 10 years of age. WT is the commonest renal tumor in the pediatric patients. The tumor is usually detected as an abdominal mass by the parents of the children.

Cytology (Figs 32.13 to 32.15)

Fine needle aspiration cytology smear of the WT shows three major components of cells: (1) blastemal cells, (2) epithelial cells and (3) stromal cells (**Box 32.8**).

Blastemal cells: These cells are predominantly discrete cells and occasionally in loose cluster. The cells are round and twice the size of the lymphocytes. The cytoplasm is scanty and deep blue forming a thin rim around the nucleus. The nuclei are round with finely dispersed chromatin and small single to multiple nucleoli.

Epithelial cells: The epithelial cells are present in cohesive clusters, small tubules or rosette-like arrangement. These so called rosettes are possibly nothing but the transversely cut tubules. The epithelial cells are larger than the blastemal cells and have moderate amount of cytoplasm. The tight three-dimensional clusters of cell resembling glomeruloid bodies are also noted.

Mesenchymal cells: These cells are oval to spindle-shaped with bland nuclei. In between the cells, deep pink collagenous material is also seen. Cells with smooth muscle or rhabdoid differentiation may be present.

Occasional cases of Wilms' tumor may show large markedly pleomorphic nuclei that usually indicate bad prognosis.

Immunocytochemistry

The blastemal cells are positive for vimentin and also show focal expression of desmin, CK and neuron specific enolase (NSE).

Differential Diagnosis

Fine needle aspiration cytology of WT may resemble the cytomorphology of small blue round cell tumor.

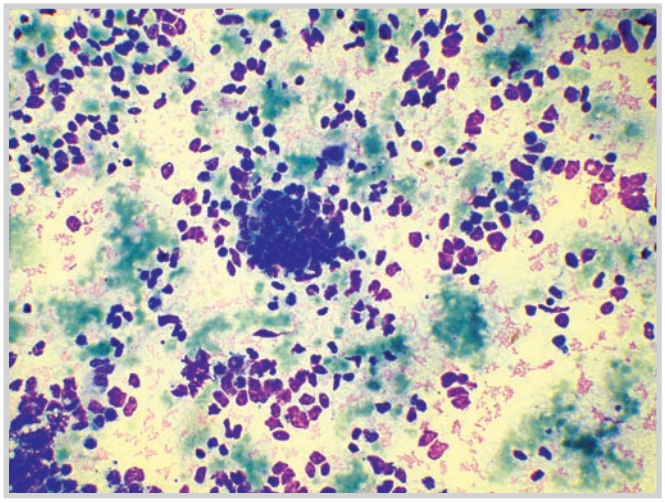

Fig. 32.13: Wilms' tumor: discrete blastemal cells and tight glomeruloid like body (MGG × MP)

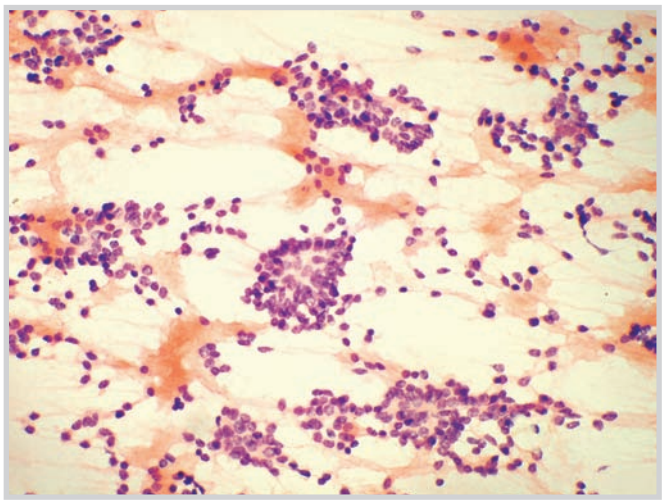

Fig. 32.14: Wilms' tumor: tubular structures and blastemal cells (H & E × MP)

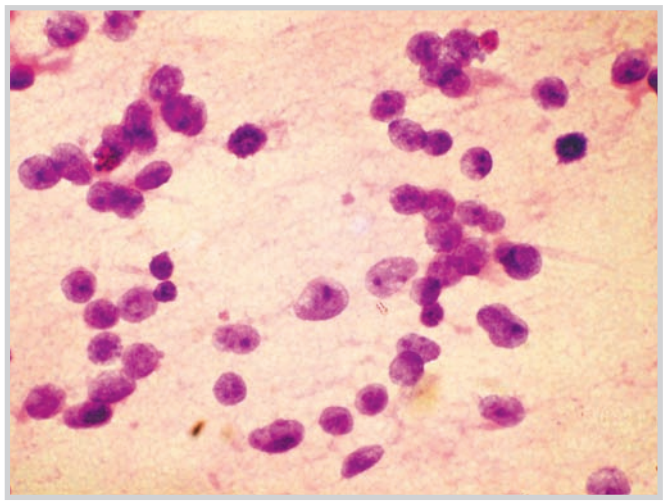

Fig. 32.15: Wilms' tumor: scanty thin rim of cytoplasm and round nuclei with finely dispersed chromatin (H & E × OI)

BOX 32.8 **Wilms' tumor**

- Small round dissociated blastemal cells
- Blastemal cells have scanty deep blue cytoplasm, round hyperchromatic nuclei
- Tubular epithelial cells
- Round well-circumscribed glomeruloid like bodies
- Spindle-shaped mesenchymal component

- *Neuroblastoma*: The blastemal cell morphology and Rosette's in neuroblastoma may simulate the tubules of WT. However, the rosettes of neuroblastoma contain neurofibrillary material and the surrounding cells are multilayered. The presence of typical mesenchymal cells may be helpful in diagnosis of WT.
- *Rhabdomyosarcoma*: Round cells with frequent binucleation and multinucleation and strap cells with cross striation are often seen in rhabdomyosarcoma. On immunocytochemistry, the cells are positive for desmin.
- *Primitive neuroectodermal tumor*: Fine needle aspiration cytology smears of primitive neuroectodermal tumor (PNET) show relatively more vacuolated cytoplasm in comparison to WT. True rosettes with central neurophilic material are seen in PNET. On immunocytochemistry, the cells are positive for synaptophysin and MIC2 and negative for WT1.

Mesoblastic Nephroma[23]

It is a low-grade fibrosarcoma of the infantile kidney. This is a congenital neoplasm and is usually detected before the age of 6 months. The infant presents with an abdominal mass.

Cytology

The FNAC smears show abundant clusters and dissociated oval to elongated spindle cells. The tumor cells show scanty cytoplasm with elongated spindle shaped bland nuclei. The nuclear chromatin is fine and nucleoli are inconspicuous. The cellular mesoblastic nephroma may show considerable nuclear pleomorphism, coarse chromatin and occasionally necrosis.[24] These cases may be mistaken as sarcoma. The cells of mesoblastic nephroma are positive for CD34 immunostaining.[25]

Differential Diagnosis

- *Congenital fibrosarcoma*: The cells of congenital fibrosarcoma show moderate nuclear pleomorphism. Nuclear chromatin is fine and often shows prominent nucleoli.

Rhabdoid Tumor

Rhabdoid tumor of kidney (RTK) is a highly invasive aggressive tumor. It represents 2% of all pediatric kidney tumors and 80% of the tumor occurs before the age of 2 years. The mean age of the patient is 1 year. RTKs are often associated with midline posterior

BOX 32.9 Rhabdoid tumor

- Discrete and small clusters
- Small cell with moderate cytoplasm
- Rhabdoid cell shows:
  - Abundant cytoplasm
  - Macronucleoli
  - Round, dense, variable sized intracytoplasmic inclusions

BOX 32.10 Clear cell sarcoma of kidney

- Clusters and discrete cells
- Polygonal cells with wispy clear cytoplasm
- Eccentrically placed grooved nuclei
- Background magenta colored mucopolysacharide material
- Prominent arborizing vessels with spindle-shaped septal cells adjacent to endothelium

fossa tumor such as medulloblastoma and hence, accurate cytological recognition is important. The patient usually presents with hematuria.

Cytology

The FNAC smear shows predominantly dispersed population and cluster of small cells.[26] Stripped bare nuclei are also seen in the background (**Box 32.9**). The individual cells are round with moderate amount of cytoplasm. The nuclei are large with irregular margin and show large prominent nucleoli. The cytoplasm shows round, dense, pinkish (MGG stain) and variably sized inclusions. This cytoplasmic inclusion is considered as one of the characteristic features of RTK.

Immunocytochemistry

The cells show strong positivity for vimentin and focal epithelial membrane antigen (EMA) positivity.

Differential Diagnosis

- *Rhabdoid differentiation of Wilms' tumor*: Rhabdoid tumor should be differentiated from the rhabdoid differentiation of WT. The rhabdomyosites of WT are large cells with abundant cytoplasm and focal cross striations.

Clear Cell Sarcoma[27]

Clear cell sarcoma of kidney (CCSK) is a rare childhood sarcoma of kidney and constitutes only 4% of all pediatric kidney tumors. It is also known as bone-metastasizing tumor as it has a propensity to metastasize in the bone. CCSK is three times more common in male child. The mean age of the patient is 3 years.

Cytology (Figs 32.16 and 32.17)

Cytology smear shows cord cells, septal cells, and arborising vasculature mucopolysaccharide substance (**Box 32.10**). The cord cells are polygonal with wispy clear cytoplasm. The nuclei are central to eccentric with deep grooves. Nuclear chromatin is fine with prominent nucleoli. Magenta colored mucopolysaccharide ground substances are often seen in the background. One important clue to diagnosis is prominent arborizing blood vessels

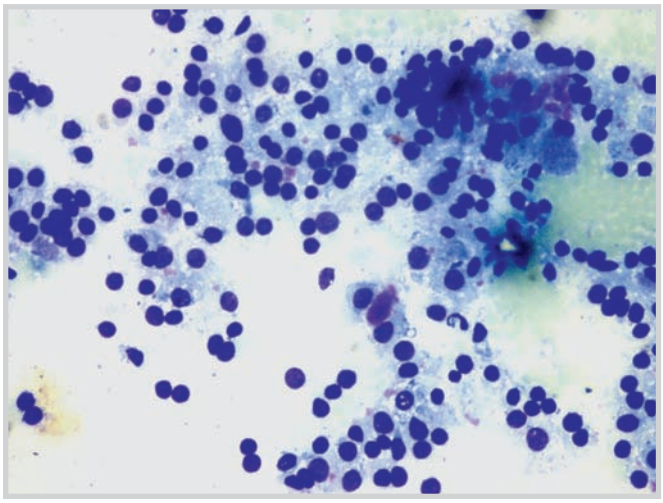

Fig. 32.16: Clear cell sarcoma of kidney: abundant discrete cells with moderate to abundant vacuolated cytoplasm (MGG × MP)

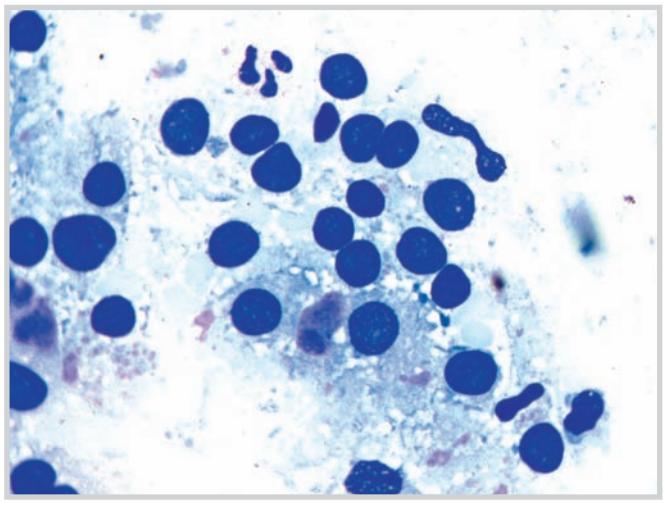

Fig. 32.17: Clear cell sarcoma of kidney: higher magnification showing cells with abundant vacuolated cytoplasm and mildly enlarged nuclei having fine chromatin (MGG × OI)

with the septal spindle-shaped cells adjacent to endothelium. In addition, dark or pyknotic apoptotic cells may also be noted. Occasionally, FNAC smears show normal tubular cells that may simulate the epithelial component of WT.

BOX 32.11 **Indications of fine needle aspiration cytology (FNAC) of adrenal**

- Recognition of metastatic tumor
- Identification of primary tumor
- Infective cases

ADRENAL

Adrenal glands are vascular retroperitoneal organ that secretes various hormones. Adrenal gland behaves as a fertile land for various tumors, both primary and secondary. Majority of these masses are asymptomatic and individuals usually present with incidental masses or are being investigated for other primary complaints. Moreover, differentiating primary adrenal cortical carcinomas, from metastatic lesions, on imaging alone, can pose a diagnostic difficulty at times. Advances in the current radiological techniques have aided in rapid diagnosis by enabling fine needle aspiration even from deep abdominal masses. The main indications of adrenal FNAC is to identify the metastatic lesions (**Box 32.11**). The primary adrenal lesions are also recognizable by FNAC. In addition, various infective organism particularly tuberculosis and fungal infections may also produce a space occupying lesions and can be confirmed by FNAC.

FINE NEEDLE ASPIRATION CYTOLOGY TECHNIQUE

Fine needle aspiration cytology of adrenal is usually done from the back side and patient should lie in prone position. The procedure is performed usually with the help of CT scan using 20–22 gauge needles. Both air dried and wet fixed smears are prepared. It is preferable to have a cell block preparation. Sample can also be collected for bacterial or fungal culture.

Fine needle aspiration cytology is usually free of major complications except minor hematuria and hypotension. However, major complications such as surgical emphysema and shock have been reported. Severe fatal hypertensive crisis may occur particularly during FNAC of pheochromocytoma due to sudden release of catecholamines.[28] The sensitivity and specificity of FNAC of adrenal are varies from 85% and 90%, respectively.[15-18] The false positive result of FNAC is rare.

ANATOMY AND HISTOLOGY

Both the adrenal glands are located in the upper pole of kidney and each weighs about 8–10 g. The gland has an outer cortex and inner medulla. The cortex is divided in three zones as zona glomerulosa, zona fasciculate and zona reticularis. The parenchymal cells of zona glomerulosa, zona fasciculate and zona reticularis synthesize and release aldosterone, cortisol, and dehydroepiandrosterone, respectively. The medullary part of adrenal gland liberates catecholamine.

The cells of the zonal glomerulosa are large with foamy cytoplasm and small round nuclei having one or two prominent nucleoli. The zona fasciculate occupies 80% of the adrenal cortex and is composed of cords of large polygonal cells with abundant vacuolated cytoplasm. The innermost layer zona reticularis constitutes only 7% of the cortex and is composed of anastomosing cords of small round cells with scanty cytoplasm.

LESIONS

Adrenocortical Neoplasm[28-31]

Adrenal neoplasm is divided into adenoma and carcinoma. It is a relatively rare tumor and commonly occurs in the middle aged person. However, the children are rarely affected by this neoplasm. The children with adrenocortical neoplasm is frequently (50% cases) associated with Li-Fraumeni syndrome.[32] The adrenal neoplasms are unilateral compared to adrenal hyperplasias which are bilateral. The majority of adrenal adenomas (85%) patients are nonfunctional, whereas most of the adrenocortical carcinomas (ACC) are functional. The tumors may be found during routine radiological investigations or they may be detected due to hormonal activities such as primary aldosteronism, Cushing's syndrome or virilization. The size of the tumor is an important criterion of malignancy and most of the ACC is larger than 6 cm diameter on radiology.

Cytology (Fig. 32.18)

On histology section, it is difficult to differentiate adrenal adenoma and carcinoma. The certain characteristic gross features of adrenal adenoma are: (1) usually less than 5 cm in diameter, (2) rarely more than 50 g in weight, (3) homogenous gross appearance without any necrosis and (4) well-encapsulated. Morphologically necrosis, high mitosis and capsular invasion are the characteristic features of ACC. However, these features are not easy to assess in FNAC smears particularly capsular invasion is not possible to assess cytologically. Therefore at times, it is difficult to distinguish adrenal adenoma from carcinoma.

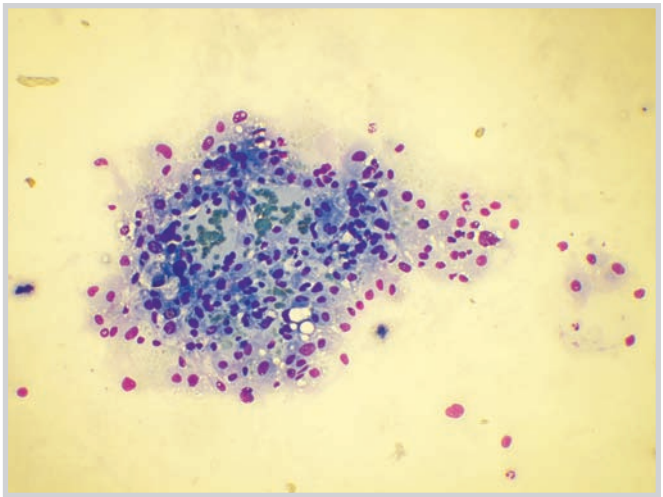

Fig. 32.18: Adrenal adenoma: cohesive cluster of cells with abundant vacuolated cytoplasm and mildly pleomorphic nuclei (MGG × MP)

BOX 32.12 Adrenal adenoma

- Bubbly lipid filled background
- Abundant round bare nuclei
- Large cohesive clusters of cells
- Vacuolated cytoplasm
- Mildly enlarged monomorphic nuclei

Fine needle aspiration cytology smears of adrenal adenoma are usually paucicellular (**Box 32.12**). The smears show three important characteristic features:

- Large cohesive clusters of cells: These cells have abundant vacuolated cytoplasm and mildly enlarged monomorphic nuclei. The cells are admixed with sinusoidal endothelial cells
- Bubbly vacuolated lipid background
- Abundant round bare nuclei.[33]

Fine needle aspiration cytology smears of ACC shows high cellularity (**Figs 32.19** to **32.21**). The cells are predominantly arranged discretely and occasional loose cohesive groups (**Box 32.13**). The individual cells are large with moderate amount of vacuolated cytoplasm and centrally placed nuclei. The nuclei are hyperchromatic with coarsely granular chromatin. Depending on the grade of ACC the nuclei show variable degree of pleomorphism. Moderately differentiated ACC may show flocculent aggregates of lipid material in the cytoplasm. Background necrosis and increased mitotic figures are noted in poorly differentiated ACC.

Immunocytochemistry

The tumor cells are positive for Melan A, calretinin, inhibin-alpha and synaptophysisn. In addition, the cells are also variably positive for cytokeratin (**Table 32.5**).

Differential Diagnosis

Renal cell carcinoma: Cytomorphologically, it is difficult to differentiate RCC from ACC. Radiological localization also may not help in renal upper pole lesions. Immunocytochemically, RCC is positive for CK and EMA (**Table 32.5**).

Pheochromocytoma[34]

This is the tumor of the chromaffin cells of the medulla of adrenal gland and is also known as paraganglioma of the adrenal gland. Pheochromocyoma is also famous as 10% tumor. About 10% of pheochromocytomas are bilateral, 10% are extradrenal and 10% malignant in nature. It may occur in all age group. The clinical symptom of pheochromocytoma is due to liberation of catecholamines. Patients often present with triad of paroxysmal hypertension, sweating and tachycardia that are virtually diagnostic of this disease.

Cytology (Figs 32.22 to 32.24)

Cytology smear of pheochromocytoma shows predominantly three types of cells (**Box 32.14**).

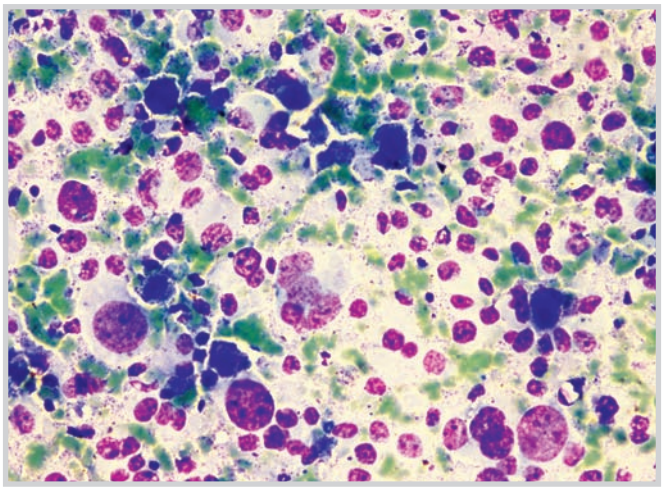

Fig. 32.19: Adrenocortical carcinoma: abundant dissociated cells in bubbly vacuolated cytoplasm (MGG × MP)

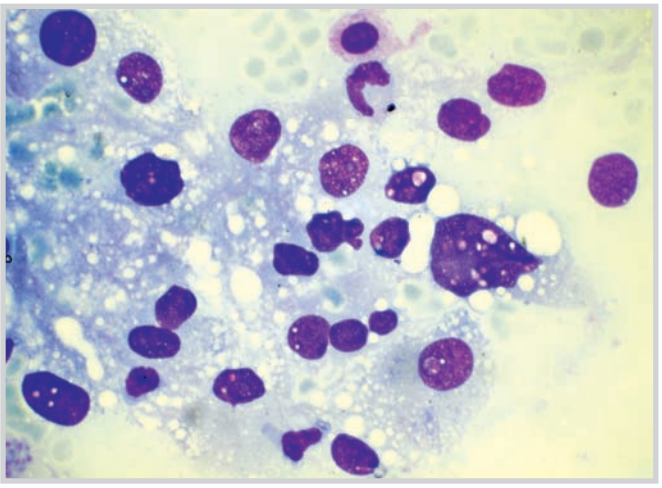

Fig. 32.20: Adrenocortical carcinoma: large cells with moderate amount of vacuolated cytoplasm and centrally placed moderately pleomorphic nuclei (MGG × HP)

- *The epithelial cell*: These cells are medium sized polygonal cells with moderate amount of reddish granular cytoplasm. Cytoplasm often contains pinkish globular bodies. The nuclei are central, round and monomorphic. The cells usually show low nucleocytoplasmic ratio. However, there may be cells with moderate nuclear enlargement and pleomorphism. Nuclear chromatin is fine with prominent nucleoli. Sudden anisonucleosis may be present.
- *Spindle cells*: These cells are spindle-shaped with elongated nuclei, coarse chromatin and abundant cytoplasm.
- *Ganglionic cells*: These cells show abundant pale cytoplasm and eccentrically placed large nuclei with prominent nucleoli.
- Nuclear enlargement and pleomorphism have no relation with the behavior of pheochromocytoma.

Differential Diagnosis

- *Adrenocortical carcinoma*: Poorly differentiated ACC often shows moderate nuclear pleomorphism and is often difficult

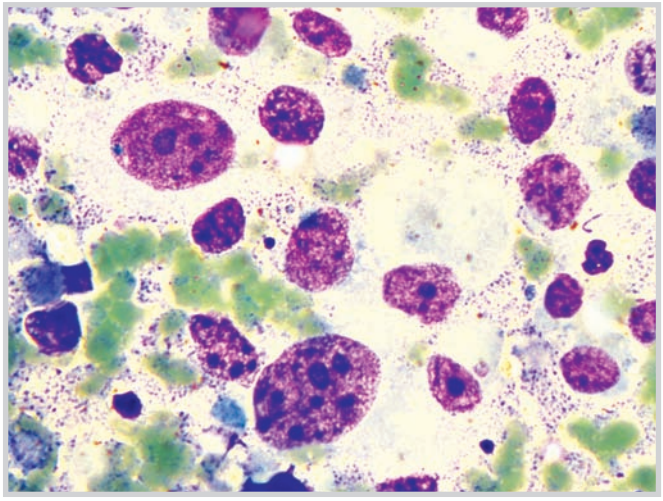

Fig. 32.21: Adrenocortical carcinoma: higher magnification showing individual cells morphology. Markedly enlarged cells with moderately pleomorphic nuclei having multiple prominent nucleoli (MGG × OI)

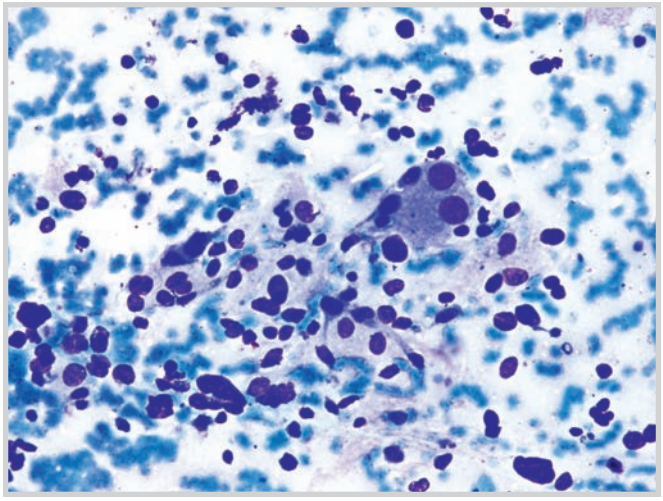

Fig. 32.22: Pheochromocyoma: discrete oval to polygonal cells and spindle cells (MGG × MP)

BOX 32.13 Adrenocortical carcinoma

- Dissociated and small cohesive clusters
- Numerous bare nuclei
- Bubbly background
- Round cells with vacuolated cytoplasm
- Centrally placed nuclei showing variable degree of pleomorphism
- Necrosis and bizarre cells

TABLE 32.5: Immunocytochemistry of renal cell carcinoma and adrenocortical carcinoma

Immunocytochemistry	*Renal cell carcinoma*	*Adrenocortical carcinoma*
Keratin AE1 and AE3	Positive	Negative
EMA	Positive	Negative
Vimentin	Positive	Positive
Melan A	Negative	Positive
Inhibin alpha	Negative	Positive
Calretinin	Negative	Positive

Abbreviation: EMA, epithelial membrane antigen

to distinguish from pheochromocytoma. The presence of reddish globular material in the cytoplasm favors pheochromocytoma. Pheochromocytoma is positive for S-100 and chromogranin.

- *Metastatic carcinoma*: Clinical history of known primary along with immunocytochemistry may be helpful to confirm the cases of metastatic carcinoma. The cells of pheochromocytoma are CK and EMA negative and positive for chromogranin.

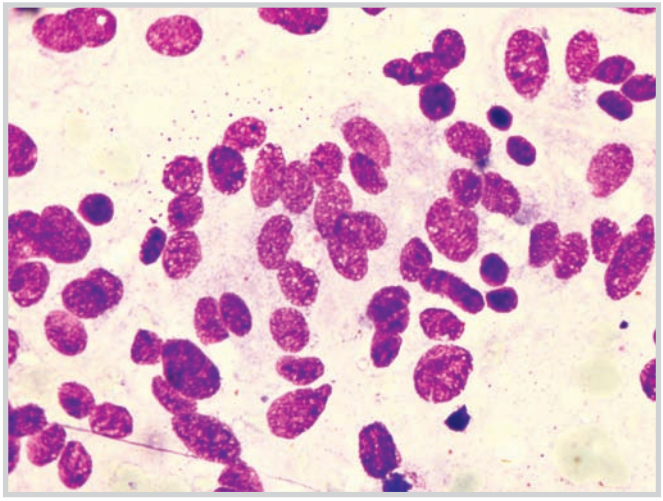

Fig. 32.23: Pheochromocyoma: oval to elongated cells with moderate pleomorphism and fine nuclear chromatin (MGG × HP)

Metastatic Tumors[35]

Metastasis in the adrenal gland is frequent and about one-fifth of cancer shows metastasis in adrenal.[36] The common primary sites of malignancies are from lung, kidney and breast. Metastatic carcinoma in the adrenal may mimic poorly differentiated ACC or pheochromocytoma. Cytomorphologically, it is very difficult to distinguish ACC from metastatic adenocarcinoma and immunocytochemistry may be helpful in this aspect (**Table 32.6**). Cells in ACC are positive for Melan A, inhibin, calretinin and negative for CK.

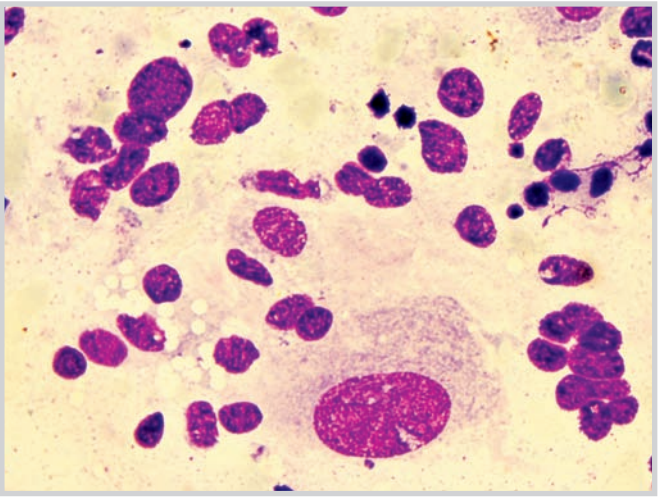

Fig. 32.24: Pheochromocyoma: sudden nuclear pleomorphism (MGG × OI)

BOX 32.14 Pheochromocytoma

- Discrete and loose clusters
- Small to moderate sized polygonal cells
- Fine reddish granular cytoplasm
- Uniform round nuclei with low nucleocytoplasmic ratio
- Spindle cells
- Occasional ganglion cell
- Sudden anisonucleosis

TABLE 32.6: Characteristic features of metastasis

Primary tumors	*Cytology*	*Immunocytochemistry*
Renal cell carcinoma	Cells with abundant clear cytoplasm	EMA, CK and vimentin
Lung carcinoma: Small cell	Small cells, scanty cytoplasm, hyperchromatic nucleus, nuclear molding	NSE, chromogranin, CK
Prostatic adenocarcinoma	Glandular differentiation	PSA
Melanoma	Large pleomorphic cells, spindle cells, cytoplasmic melanin pigment	HMB45
Colorectal adenocarcinoma	Glandular differentiation	CK20 positive and CK7 negative
Breast carcinoma	Malignant ductular cells.	CK7 positive, CK20 negative. ER, PR, and GCDFP-15 positive

Abbreviations: EMA, epithelial membrane antigen; CK, cytokeratin; NSE, neuron specific enolase; PSA, prostate-specific antigen; ER, estrogen receptor; PR, progesterone receptor; GCDFP gross cystic disease fluid protein

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CHAPTER 33

Gonads and Prostate

Chapter Contents

TESTIS

Testis is the male gonad situated within the scrotal sac. Fine needle aspiration cytology (FNAC) of the testis is now a well-established technique. It is mainly done to investigate the nature of the swelling and also to investigate the cases of male infertility. There is an unproven risk of dissemination of malignancy by FNAC through the needle tract. Therefore, many people doubt the necessity of testicular FNAC. In our experience in Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh (India), we did not encounter any case of dissemination of malignancy through needle tract during testicular FNAC. We have not faced any case of local recurrence or inguinal metastasis due to FNAC of testis. FNAC of the testis is a rapid, economic and reliable procedure and provides the important information about the nature of the lesion. For the surgeon, Fine needle aspiration biopsy (FNAB) of testicular masses is a helpful guide in selecting the best operative procedure. An accurate preoperative diagnosis is important in determining the correct surgical approach for the patient. FNAC of the scrotal swelling is, however, less controversial and it can be used to diagnose the various cysts and inflammatory lesions.

Anatomy and Histology of Testis

Each testis is approximate 4 cm long and 2–3 cm wide. Testis develops in the abdominal cavity and then descends in the scrotal sac dragging a part of peritoneum known as tunica vaginalis.

Testis is composed of numerous seminiferous tubules that are surrounded by richly vascular loose connective tissue. In the connective tissue, there are small conglomerations of endocrine cells present which are known as the interstitial cells (of Leydig). Within the seminiferous tubule the germ cells undergo maturation through various stages: spermatocytes to spermatids to spermatozoa. The mature spermatozoa travel from seminiferous tubules to rete testis to ductus efferens and finally stores in epididymis.

Normal Cells of Testis—Fine Needle Aspiration Cytology (Fig. 33.1)

Sertoli cells: These are relatively large cells with fragile cytoplasm and indistinct cell border. The cytoplasm may show fine vacuolations and spermatozoa. The nucleus of Sertoli cells is round to oval, with granular chromatin and a prominent nucleolus.

Spermatocytes: The primary spermatocytes show scanty, deeply basophilic cytoplasm with round to oval nuclei having fine-thread-forming or coarse clumped chromatin. Nucleoli are indistinct. Secondary spermatocytes are not identified.

Spermatids: These cells are often present in small groups. The cytoplasm of the cell is scanty and vacuolated. The nuclei are round with clumped chromatin and indistinct nucleoli.

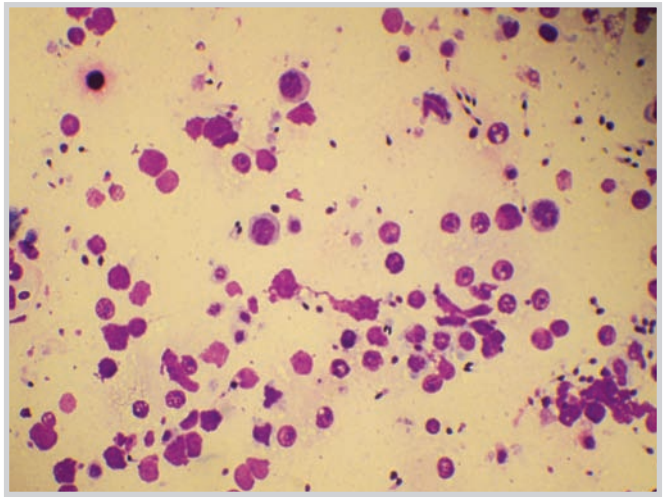

Fig. 33.1: Normal testicular tissue: Sertoli cells, spermatocytes, spermatids and spermatozoa [May-Grünwald-Giemsa (MGG) stain × high power (HP)]

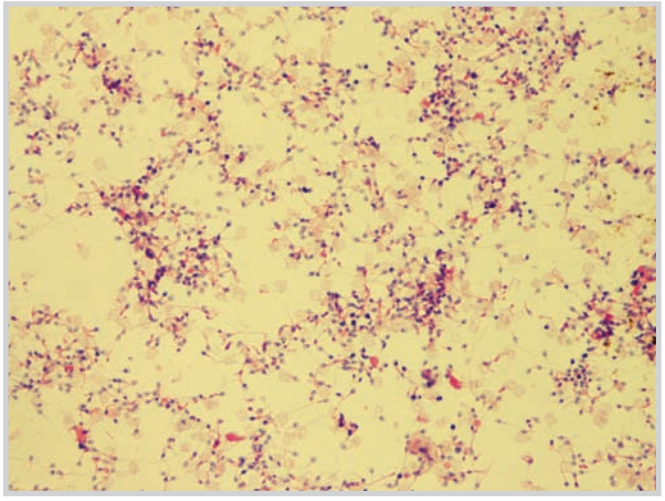

Fig. 33.2: Spermatocele: Abundant spermatozoa in the centrifuged fluid of the spermatocele [hematoxylin and eosin (H & E) stain × medium power (MP)]

Spermatozoa: These are triangular-shaped cells with eccentric nuclei. Chromatin of the nuclei is condensed. The tip of the spermatozoa shows a clear cytoplasmic outline. Sperm shows a long tail.

Leydig cells: These are large cells with abundant fine pink granular cytoplasm.

The nuclei are centrally placed nuclei with fine granular chromatin and prominent nucleoli. Crystalloids of Reinke are occasionally seen as unstained areas in the background in May-Grunwald Geimsa (MGG)-stained smears.

PERITESTICULAR LESIONS

Hydrocele

Hydrocele is the accumulation of fluid in the tunica vaginalis. Hydrocele may be congenital or acquired. The acquired hydrocele is often associated with inflammation of the tunica vaginalis. Aspirates of hydrocele yields clear straw colored fluid and smears show many sheets of benign tunica lining mesothelial cells.

Spermatocele

Spermatocele is the cystic dilatation of the efferent ducts (**Fig. 33.2**). The cyst is filled with spermatozoa. This is usually associated with a history of local operation such as vasectomy etc. FNAC smears show many spermatozoa and foamy macrophages with ingested sperms.

Inflammation

Tuberculous epididymitis: Tuberculosis of the epididymitis is common in the Indian subcontinent. The aspirate usually yields thick cheesy material. The FNAC smear shows multiple epithelioid cell granulomas, multinucleated giant cells and lymphocytes on a necrotic background. Ziehl-Neelsen(Z-N) stain for acid fast bacilli (AFB) is positive in half of such cases.

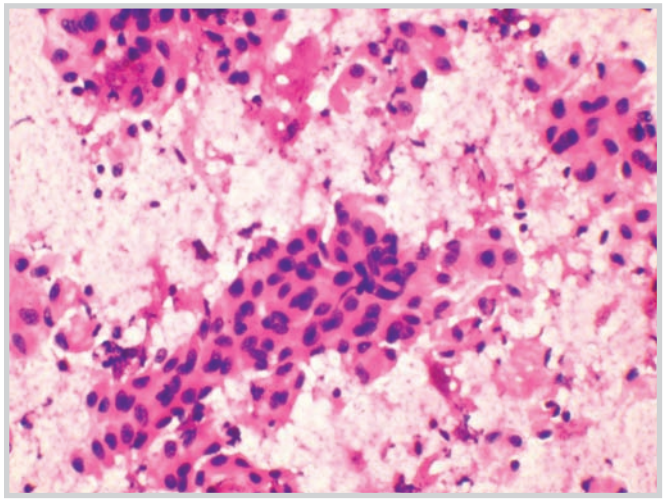

Fig. 33.3: Adenomatoid tumor: Multiple clusters and discrete monomorphic epithelial cells (H & E × HP)

Other causes of granulomatous epididymitis: There are other causes of granulomatous inflammation in epididymitis such as fungal infection, granulomatous ischemic lesion and idiopathic granulomatous epididymitis.

Filarial inflammation: Microfilarial infestation in the epididymis often provokes chronic inflammation. Cytology smear shows microfilaria along with variable amount of multinucleated giant cells and granulomas. It is preferable to screen the smear in lower magnification to detect such parasites.

Adenomatoid tumor:[1] This is the commonest tumor of the epididymis and occurs in third and fourth decade of life. The cytology smear shows multiple clusters and sheets of monotonous population of cells (**Fig. 33.3**). The individual cells are round to oval with round monomorphic nuclei. Nuclei show fine chromatin and small inconspicuous nucleoli. Discrete spindle cells are also seen.

The tumor cells show strong reactivity for cytokeratin (CK), epithelial membrane antigen (EMA), calretinin, and Wilms' tumor 1(WT1). The cells are negative for carcinoembryonic antigen (CEA) and factor VIII (FVIII)-related antigen.[2]

Spermatocytic Granuloma[1]

Spermatocytic granuloma presents as a nodule in the head of the epididymitis (**Figs 33.4** and **33.5**). FNAC smears show mixed inflammatory cells consisting of lymphocytes, macrophages and occasional ill-formed epithelioid cell granulomas. The background of the smear usually contains many spermatozoa or sperm heads. Macrophages with ingested sperm heads are frequently seen. Z-N stain for AFB is always negative in this granulomatous inflammation.

Evaluation of Male Infertility[3-7]

In 1965, Obrant and Persson described FNAC for getting material for cytological evaluation of spermatogenesis.[7] Long time testicular FNAC for evaluation of male infertility was not popular as many clinicians were afraid of trauma and hemorrhage. However, FNAC of the testis is almost free of complications. FNAC is a relatively easy and quick procedure to assess any obstruction in the efferent duct system and the status of spermatogenesis. The various cells in maturation are easily identified in FNAC smears. FNAC of testis has almost 100% diagnostic accuracy in the evaluation of male azoospermic patient.[6]

Sertoli cell only syndrome (**Figs 33.6 and 33.7**): The smears show large number of sertoli cells. No other cells are seen. Smear may also show variable number of mast cells.

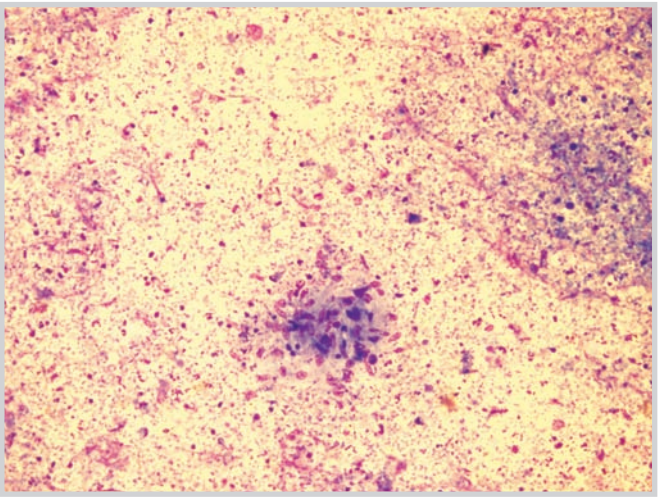

Fig. 33.4: Spermatocytic granuloma: Epithelioid cell granulomas along with many spermatozoa in a dirty necrotic background (MGG × MP)

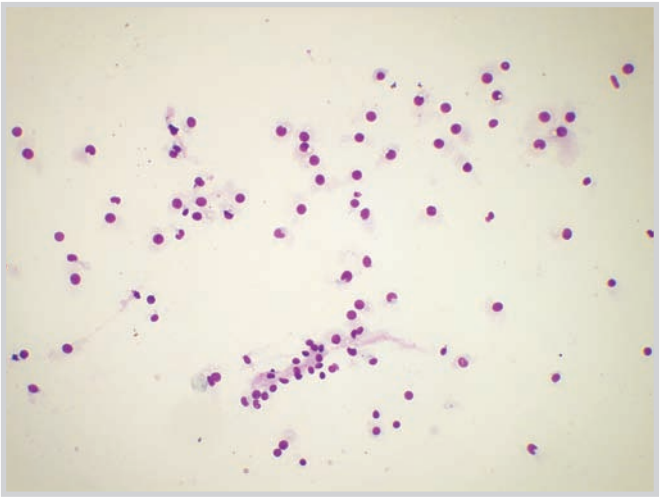

Fig. 33.6: Sertoli cell only syndrome: Only Sertoli cells present (MGG × MP)

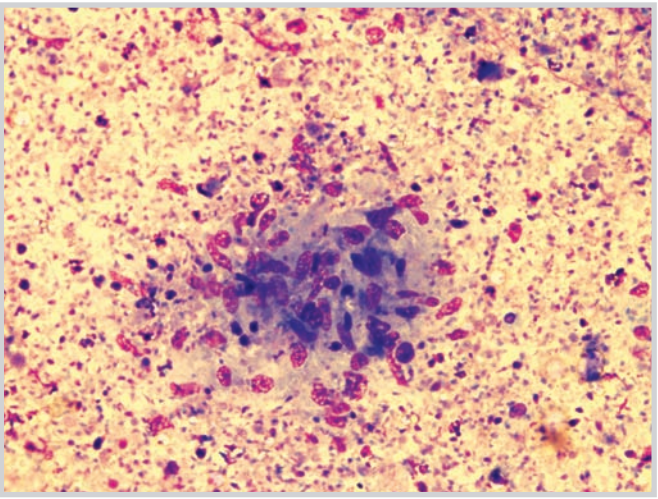

Fig. 33.5: Spermatocytic granuloma: Higher magnification of the epithelioid cell granuloma (MGG X HP)

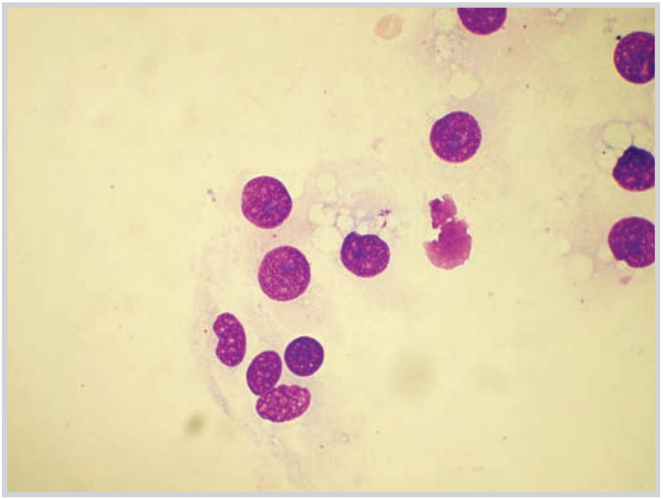

Fig. 33.7: Sertoli cell only syndrome: Higher magnification showing Sertoli cells with abundant cytoplasm having indistinct border (MGG × OI)

Early maturation arrest: Fine needle aspiration cytology smears show spermatocytes and sertoli cells in early maturation arrest. No spermatids or sperm are seen.

Late maturation arrest: The smears show sertoli cells, spermatocytes and spermatids; however no sperm is seen.

In case of normal spermatogenesis, all types of cells are seen. The presence of normal maturation up to the level of spermatozoa in FNAC smear in an azoospermic male indicates the possibility of obstruction in the pathway of semen.

NEOPLASM OF TESTIS

Testicular tumor is classified by World Health Organisation (WHO)[8] as (**Box 33.1**):

Seminoma [9-13]

This is the most common germ cell tumor of testis and represents about one third of all testicular tumors. The incidence of this tumor increases sharply after puberty. It commonly occurs between 30–40 years of age. The patient usually presents as painless enlargement of testis.

BOX 33.1 Testicular tumor classification (modified WHO)

Germ cell tumors
- Intratubular germ cell neoplasia, unclassified
- Other types

Tumors of one histological type (pure forms)
- Seminoma
- Spermatocytic seminoma
- Embryonal carcinoma
- Yolk sac tumor
- Trophoblastic tumors
- Choriocarcinoma
- Trophoblastic neoplasms other than choriocarcinoma
  - Monophasic choriocarcinoma
  - Placental site trophoblastic tumor
- Teratoma
  - Dermoid cyst
  - Monodermal teratoma
  - Teratoma with somatic type malignancies

Tumors of more than one histological type (mixed forms)

Sex cord/gonadal stromal tumors
- Leydig cell tumor
- Malignant Leydig cell tumor
- Sertoli cell tumor
- Malignant sertoli cell tumor
- Granulosa cell tumor

Tumors of the thecoma/fibroma group
- Thecoma
- Fibroma
- Sex cord/gonadal stromal tumor:
- Others

Miscellaneous tumors of the testis: Carcinoid, Paraganglioma, etc.

Cytology (Figs 33.8 and 33.9)

There are two histological types of seminoma: (1) classical and (2) spermatocytic. The classical seminoma shows discrete malignant cells in a typical lace-like vacuolated background known as tigroid background (**Box 33.2**). This is due to the cytoplasmic contents of the fragile cells. The individual cells are round to oval in shape and three to four times larger than the mature lymphocytes. The cells show scanty to moderate amount of vacuolated cytoplasm. Nuclei are round, mildly pleomorphic with fine granular chromatin and prominent nucleoli. The fragile nucleus often gives rise to thin long chromatin threads. Cytoplasmic fragments of the cells resembling lymphoglandular

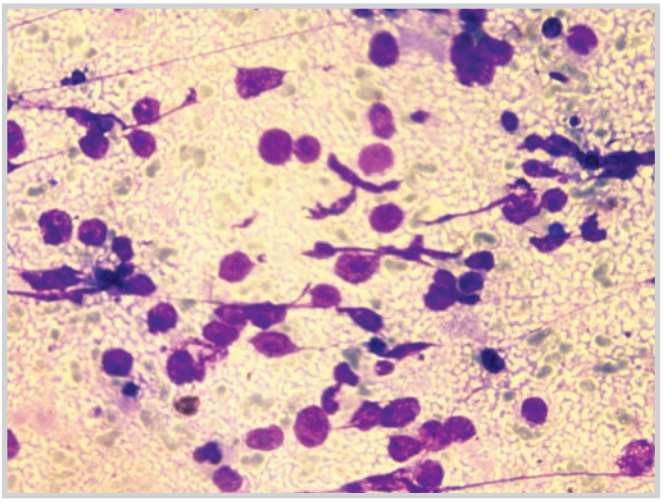

Fig. 33.8: Seminoma: Discrete round cells in a tigroid background (MGG × MP)

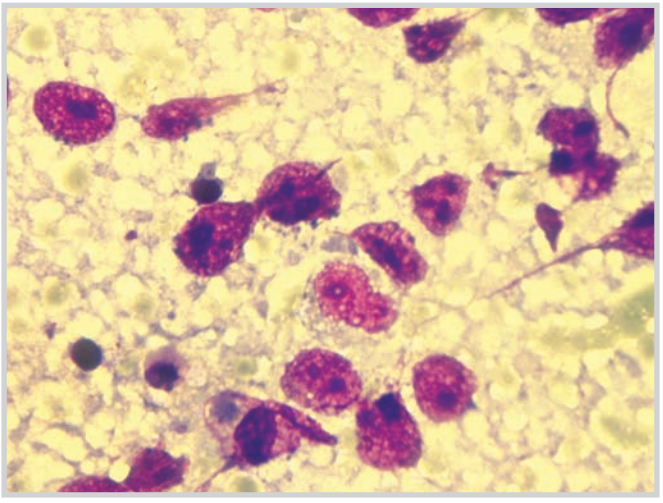

Fig. 33.9: Seminoma: Round cells with vacuolated cytoplasm. Nuclei are enlarged with fine chromatin and prominent nucleoli [(MGG × oil immersion(OI)]

BOX 33.2 Seminoma

- Discrete cells
- Tigroid lacy vacuolated background
- Scanty vacuolated cytoplasm
- Round mildly pleomorphic nuclei, fine chromatin, prominent nucleoli
- Background lymphocytes
- Epithelioid cell granulomas

Immunocytochemistry: Positive for placental alkaline phosphatase (PLAP) and CD117 (C-Kit)

bodies are also noted. In addition, the mature lymphocytes are also seen in the background. Epithelioid cell granulomas are occasionally noted in case of seminoma of testis. Seminoma is positive for placental alkaline phosphatase (PLAP) and CD117. The cells are negative for high molecular weight keratin, EMA and CD30.

Spermatocytic seminoma represents only 4% of all seminoma. They show variable population of cells consisting of small, medium and large cells. The medium sized cells usually are the dominant population. These cells show scanty to moderate cytoplasm and large central nuclei with coarse nuclear chromatin and prominent nucleoli. Small cells show scanty deep blue cytoplasm with irregular nuclei having dense chromatin. The large cells have abundant cytoplasm and moderately pleomorphic large nuclei. The cells often are binucleated with multiple prominent nucleoli.

Unlike classical seminoma, the spermatocytic seminoma shows a clean background that is free of any lymphocytes. Tigroid lacy vacuolated background is absent in such tumor.

Differential Diagnosis

- *Nonseminomatous germ cell tumor:* The other types of germ cell tumors may simulate seminoma. The typical tigroid background, lymphocytes and individual cell morphology may be helpful. Frequent admixture of different germ cell tumors may pose significant problem in diagnosis.
- *Non-Hodgkin lymphoma (NHL):* NHL shows lack of tigroid background. Lymphoglandular bodies are always present in NHL. NHLs are positive for CD45 and negative for PLAP.
- *Metastatic carcinoma:* Metastatic carcinoma shows cohesive clusters of malignant cells with moderate nuclear enlargement and pleomorphism. The cells are positive for CK and negative for leukocyte-common antigen (LCA) and PLAP.

Embryonal Carcinoma

The cytological features of embryonal carcinomas are mentioned in **Box 33.3**.

BOX 33.3 Embryonal carcinoma

- Cohesive three-dimensional clusters
- Small acinar, tubular and papillary pattern
- Large cells, moderately pale vacuolated cytoplasm
- Large moderate to marked pleomorphic nuclei
- Single to multiple prominent nucleoli, often macronucleoli

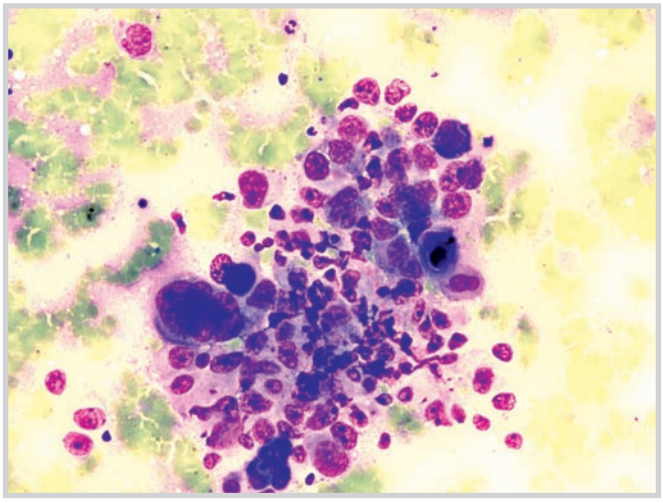

Fig. 33.10: Embryonal carcinoma: Loose cluster of cells with markedly pleomorphic cells (MGG × HP)

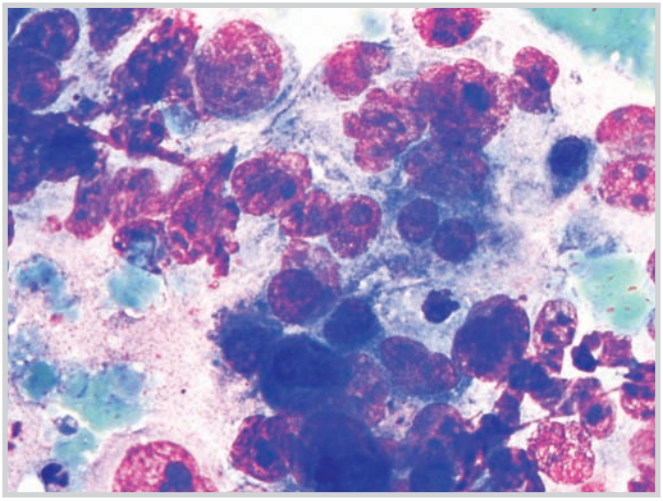

Fig. 33.11: Embryonal carcinoma: Large cells with enlarged nuclei having multiple prominent nucleoli (MGG × OI)

Cytology (Figs 33.10 and 33.11)

The aspirates of embryonal carcinoma show predominantly cohesive clusters of cells along with acinar, tubular and papillary arrangement. The tumor cells show large highly pleomorphic nuclei with irregular nuclear margin. The nuclei contain multiple prominent nucleoli. Background of the smear may show necrosis. The tumor cells are positive for AFP, high molecular weight keratin, CD30 and negative for CD117.

Yolk Sac Tumor

Yolk sac tumor is also called as endodermal sinus tumor. The tumor may occur in pure form or as a component of mixed

germ cell tumor. The smear shows multiple cohesive clusters of cells in a mucoid background. The tumor cells are moderately pleomorphic with vacuolated cytoplasm. Nuclei show multiple prominent nucleoli. Intracytoplasmic and extracellular pink globules may also be noted. These globules are periodic-acid Schiff (PAS) positive and diastase resistant. The cells of endodermal sinus tumor are positive for PLAP and AFP.

Choriocarcinoma

Choriocarcinoma represents 5% of all testicular tumors. The tumor is usually small in size and there may not be any enlargement of testis. The cytology smear shows syncytio and cytotrophoblasts. The syncytiotrophoblasts are relatively large cells with abundant cytoplasm and large severely pleomorphic nuclei with multiple prominent nucleoli. The cytotrophoblastic cells are small sized with scanty vacuolated cytoplasm having round mildly pleomorphic nuclei and indistinct nucleoli. Background of the smear usually shows large amount of necrosis. The cells are consistently positive for human chorionic gonadotropin (hCG) and keratin.

Other Tumors of Testis

Non-Hodgkin lymphoma of the testis represents only 2% of all the testicular tumors. NHL of testis usually affects the elderly patient. Most of the testicular lymphomas are diffuse large B-cell lymphoma (DLBCL). The cytomorphology of NHL is similar as that of other areas (**Fig. 33.12**).

FEMALE GENITAL SYSTEM

Many clinicians do not recommend FNAC of frankly malignant operable ovarian tumor because of possible rupture and spillage of cyst material to the peritoneal cavity. Moreover, the preoperative diagnosis of malignant ovarian tumor may not be needed because the surgical excision may be the treatment of choice. FNAC of ovarian tumor is indicated in:

- Confirmation of recurrence of the malignant tumor
- To diagnose advanced un-resectable ovarian tumor
- Confirmation of benign incidental ovarian tumor

Fine needle aspiration cytology of ovarian tumor is done under ultrasonography (USG) or computed tomography (CT) scan guidance. The benign cyst in USG is usually thin-walled, simple cyst without any septation. No solid areas are seen. FNAC from the ovary is almost free of any complications. In our institution, we have never seen any needle tract seeding after FNAC of ovarian malignancy. Due to heterogeneity of the ovarian histology in various parts of the ovarian mass, it is not always possible to have proper sampling from the representative area and malignancy may be underdiagnosed. The sensitivity of ovarian tumor is as high as 90%. The false negative FNAC report is particularly seen in case of borderline tumors.

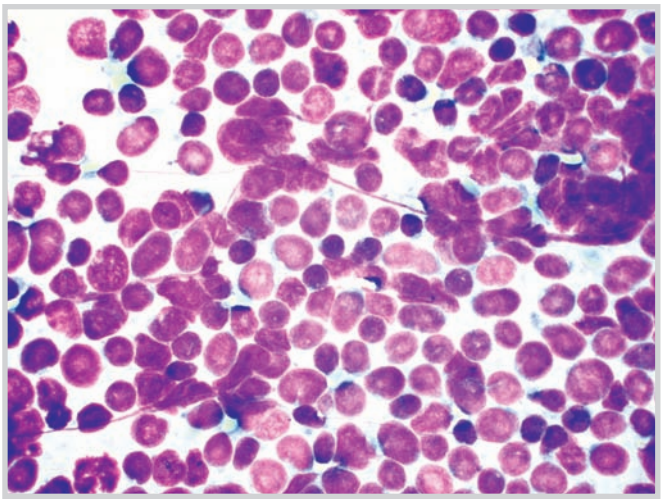

Fig. 33.12: Non-Hodgkin lymphoma (NHL) testis: Lymphoblastic lymphoma secondarily involved in testis (MGG × OI)

Non-neoplastic Cyst

Follicular Cyst

Follicular cyst of ovary may be of variable sized and unilateral. USG shows unilocular thin-walled fluid filled cyst. The majority of them spontaneously regressed within a few months.

Fine needle aspiration cytology of the cyst yields clear fluid. The smear of the fluid is usually paucicellular. However, occasionally the smears may be hypercellular. The cells are discrete granulosa cells with scanty to moderate amount of vacuolated cytoplasm and round monomorphic nuclei. The nuclei show granular chromatin and 1–2 prominent nucleoli. Nuclear grooves may be seen in occasional cells.

Corpus Luteal Cyst

This is due to persistent presence of corpus luteal cyst. The cyst is usually unilocular and lined by corpus luteal cell.

Cytology smear of the corpus luteal cyst shows discrete corpus luteal cells (**Box 33.4**). The cells are large with abundant finely vacuolated cytoplasm. Nuclei are central and pyknotic. In addition, the smear may show golden brown hemosiderin laden histiocytes.

Endometriotic Cyst

Fine needle aspiration cytology smears of the endometriotic cyst show hemosiderin laden histiocytes and occasional

BOX 33.4 Corpus luteal cyst

- Large cells
- Abundant finely vacuolated cytoplasm
- Central pyknotic nuclei
- Hemosiderin laden histiocytes

BOX 33.5 Endometriotic cyst

- Hemosiderin laden histiocytes
- Endometrial cells
- Stromal cells: Round with moderate cytoplasm

degenerated endometrial glandular cells and endometrial stromal fragments (**Box 33.5**). The endometrial cells are arranged in small clusters. The cells are small with scanty cytoplasm. The nuclei are monomorphic hyperchromatic with inconspicuous nucleoli. The endometrial stromal cells are round with moderate cytoplasm and oval nuclei.

Ovarian Tumor[14-18]

Serous and Mucinous Adenocarcinoma (Figs 33.13 to 33.15)

The cytology smears of serous adenocarcinoma show multiple cohesive clusters and discrete cells. Smears may also show multiple glands or papillary arrangement of cells. The malignant cells are round to oval with moderate amount of vacuolated cytoplasm (**Box 33.6**). The nuclei show moderate pleomorphism with vesicular chromatin having prominent nucleoli. In addition, psammoma bodies are also seen. Tumor necrosis and increased mitotic activities are the indicators of malignancy. At times, it is very difficult to distinguish a borderline serous cystadenoma from the serous cystadenocarinoma. In difficult case, histopathology is needed.

Mucinous Cystadenocarcinoma

Fine needle aspiration cytology smears of mucinous adenocarcinoma show multiple three-dimensional cohesive clusters of malignant cells in the background of mucinous material (**Box 33.7**). The individual cells are columnar in shape with central to eccentric basally placed nuclei. The cytoplasm of the cell is moderately vacuolated. The nuclei are large with mild to moderate pleomorphism and prominent nucleoli.

Differential Diagnosis

- *Metastatic carcinoma:* Metastatic carcinoma particularly metastatic colonic carcinoma is difficult to differentiate from the primary ovarian carcinoma.
- *Borderline malignancy:* It is impossible to differentiate a borderline ovarian malignancy from a frank malignancy in FNAC.

Endometrioid Carcinoma

On Fine needle aspiration cytology smear, it is difficult to differentiate endometrioid carcinoma from serous adenocarcinoma. The smears show discrete cells and small clusters or fragments of cells. The cells are columnar in shape with moderately pleomorphic nuclei. Background may show hemorrhage, necrosis and hemosiderin laden macrophages.

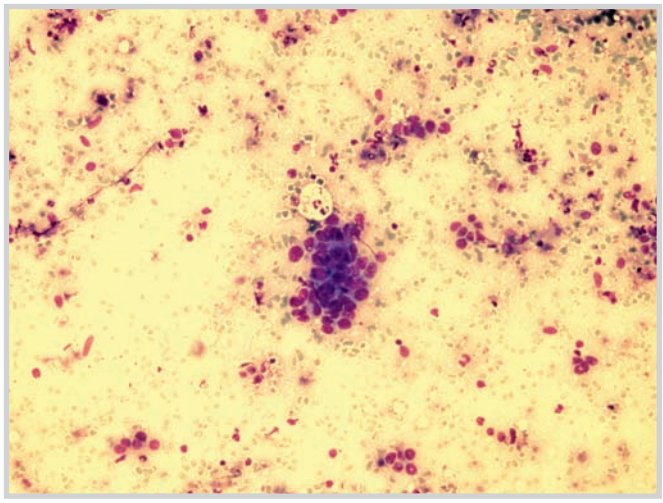

Fig. 33.13A: Serous cystadenocarcinoma: Cohesive cluster and discrete malignant cells (MGG × MP)

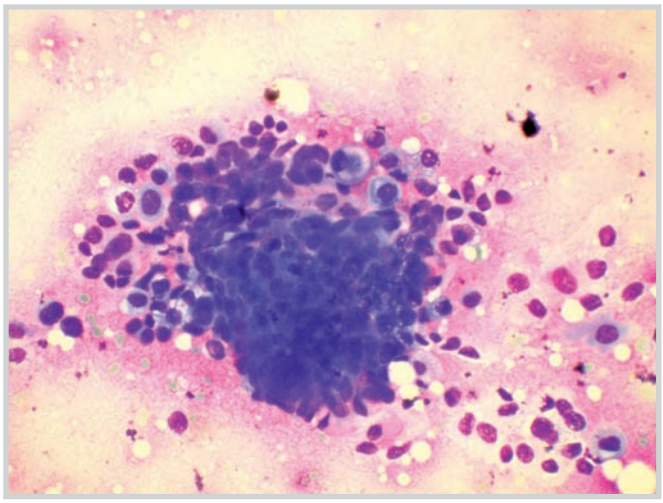

Fig. 33.13B: Serous cystadenocarcinoma: Round cells with moderately pleomorphic enlarged nuclei (MGG × HP)

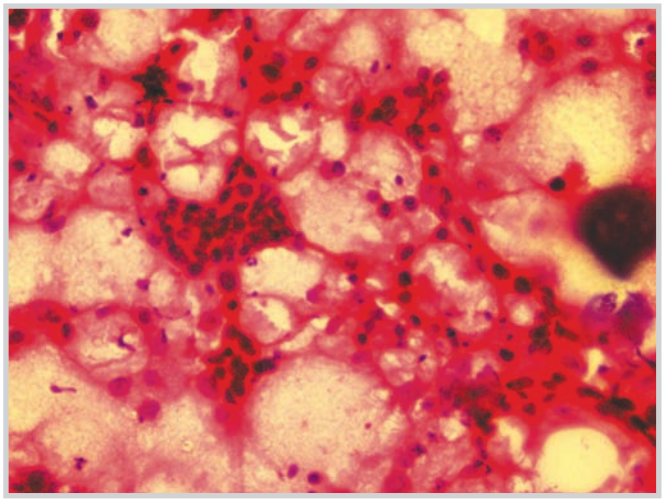

Fig. 33.14: Serous cystadenocarcinoma: Clusters and discrete malignant epithelial cells seen (H & E × MP)

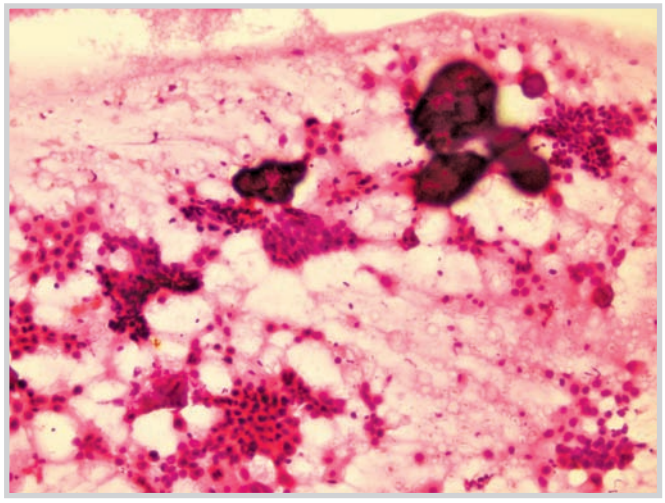

Fig. 33.15A: Serous cystadenocarcinoma: Concentric laminated psammoma body (H & E × MP)

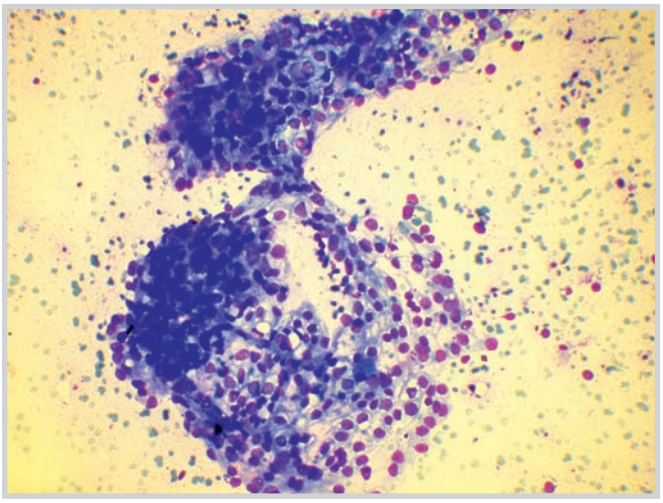

Fig. 33.16: Clear cell carcinoma: Cluster of cell with clear vacuolated cytoplasm (MGG × MP)

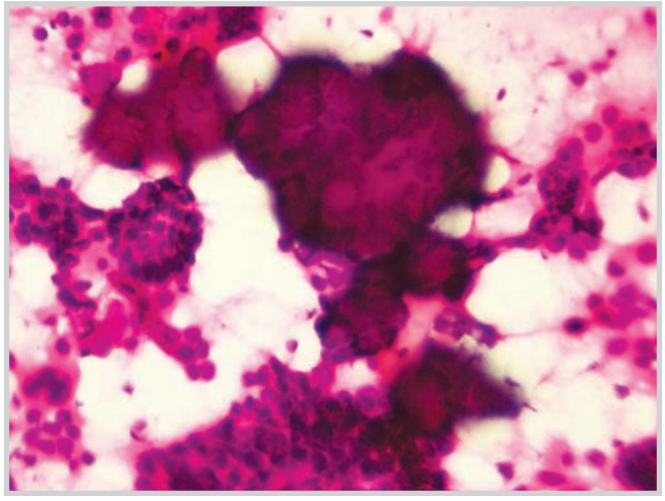

Fig. 33.15B: Serous cystadenocarcinoma: Higher magnification of the psammoma bodies (H & E × HP)

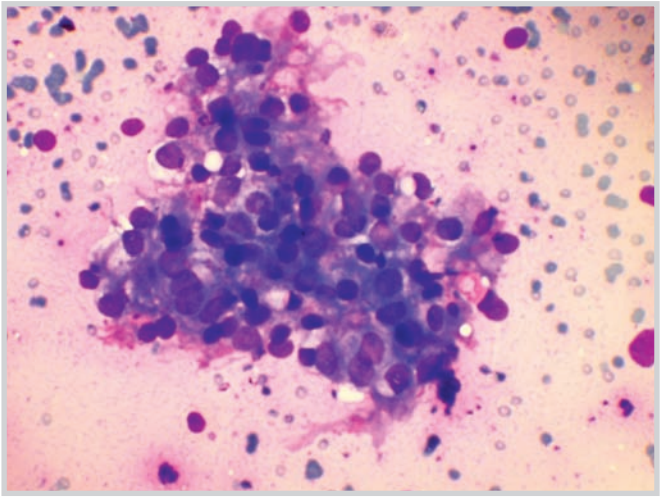

Fig. 33.17: Clear cell carcinoma: Higher magnification showing round to oval cells with moderate amount of vacuolated cytoplasm and enlarged nuclei (MGG × HP)

BOX 33.6 **Serous cystadenocarcinoma**

- Cohesive clusters, single cells and glandular arrangement
- Large round cells
- Pleomorphic nuclei
- Prominent nucleoli
- Psammoma bodies

BOX 33.7 **Mucinous cystadenocarcinoma**

- Mucinous material
- Columnar cells
- Intracellular mucin
- Pleomorphic nuclei
- Prominent nucleoli

Clear Cell Carcinoma (Figs 33.16 to 33.18)

Fine needle aspiration cytology smear shows clusters and discrete tumor cells. These cells have abundant clear pale vacuolated cytoplasm with centrally placed moderately pleomorphic nuclei (**Box 33.8**). The nuclei show frequent binucleation and prominent nucleoli.[19] Extracellular hyaline material may also be noted.

Differential Diagnosis

- *Metastatic renal cell carcinoma:* The cells of clear cell carcinoma of ovary may simulate metastatic renal cell carcinoma.

Germ Cell Tumor

Cytomorphology of germ cell tumor is similar to that of testicular germ cell tumor. This is already described before.

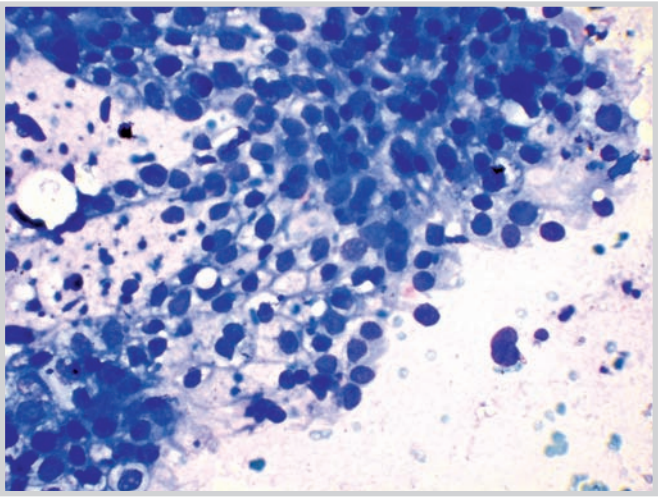

Fig. 33.18: Clear cell carcinoma: Cells with clear vacuolated cytoplasm and moderately pleomorphic nuclei (MGG × HP)

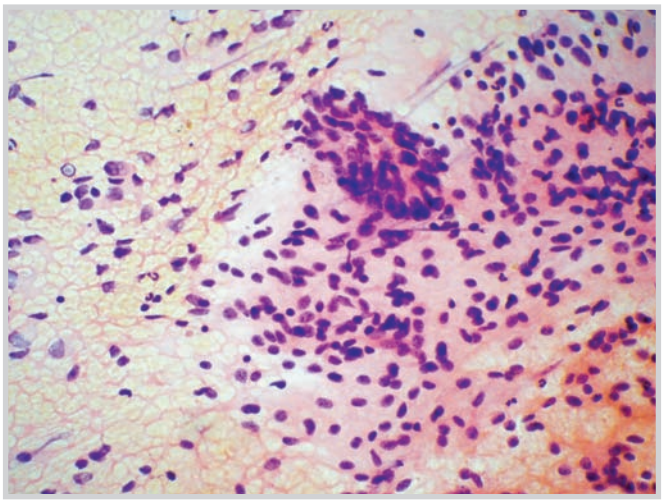

Fig. 33.19: Immature teratoma: Discrete round to oval cells and loose cluster of spindle cells embedded in connective tissue (H & E × MP)

BOX 33.8 Clear cell carcinoma

- Clear vacuolated cytoplasm
- Central nucleus
- Moderate nuclear pleomorphism

Mature Teratoma

Fine needle aspiration cytology smears of mature teratoma yield pultaceous material. Smears show many aneucleated squamous cells, hair follicles, sebaceous cells, ciliated columnar epithelial cells and intestinal epithelial cells. In addition, mesenchymal and mature neural components may be seen.

Immature Teratoma (Figs 33.19 to 33.22)

Immature teratoma is an intermediate type of malignancy and graded in histopathology according to the presence of immature neural elements. In addition to mature components, the FNAC smears show immature neural components such as rosette-like structures and round cells simulating cells of neuroblastoma. These cells show scanty cytoplasm, round nuclei and fine chromatin. Occasionally, the tumor shows other germ cell tumors particularly yolk sac tumors. The presence of hyaline globules and large cells with severely pleomorphic nuclei having multiple prominent nucleoli suggests the possibility of yolk sac tumors. Serum AFP is elevated in such cases.

Dysgerminoma

Cytomorphological features of dysgerminoma are similar to that of seminoma of the testis. The tumor cells are predominantly discrete in a tigroid background. The cells are large round with scanty vacuolated cytoplasm and mildly pleomorphic nuclei with prominent nucleoli. Mature lymphocytes are admixed with the tumor cells.

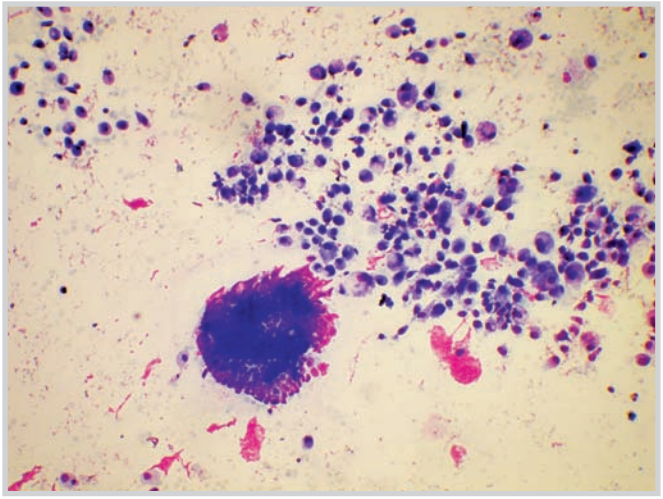

Fig. 33.20: Immature teratoma: Discrete columnar cells and cluster of round cells (MGG × MP)

Granulosa Cell Tumor (Figs 33.23 to 33.25)

This tumor develops from the granulosa cells of ovary and represents 1% of all ovarian tumors. The tumor occurs in all age group. The functional granulosa cell tumor may show hyperestrogenic symptoms such as irregular bleeding in post menopausal woman, menstrual disorders in reproductive age group and pseudoprecocious puberty in prepubertal girl.

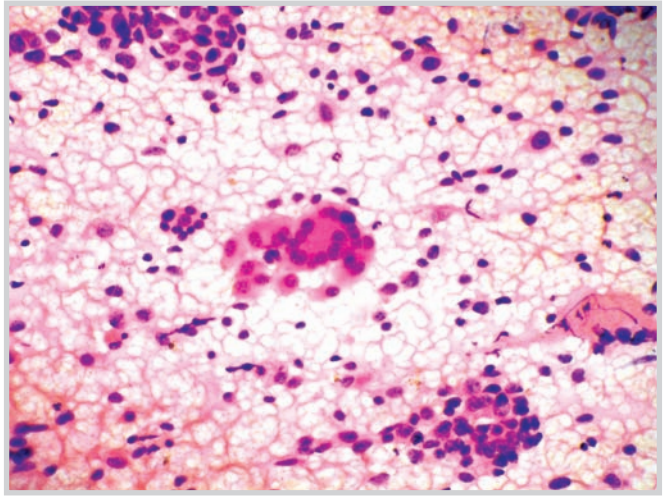

Fig. 33.21: Immature teratoma: Round discrete cells and tubules (H & E × MP)

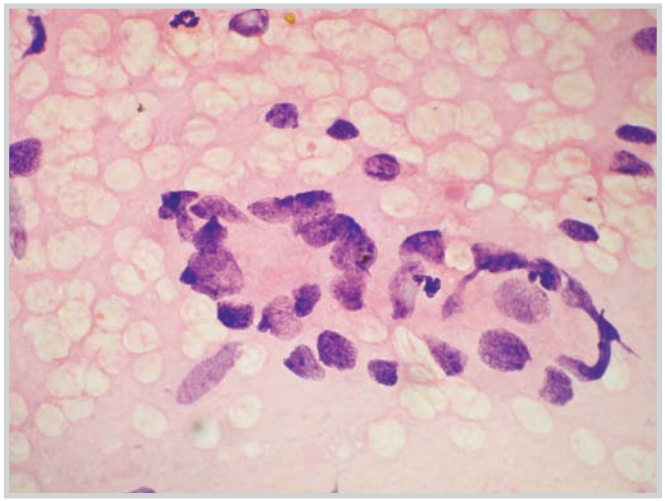

Fig. 33.22: Immature teratoma: Vague rosette-like structure (H & E × HP)

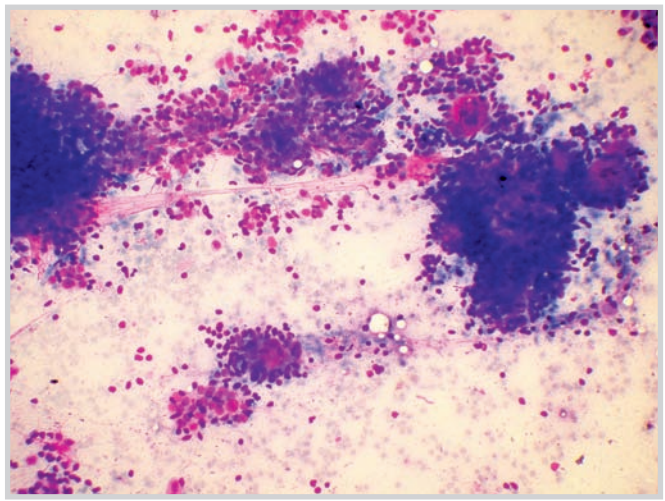

Fig. 33.23: Granulosa cell tumor: Monomorphic round cells in clusters and arranged around reddish globules (MGG × MP)

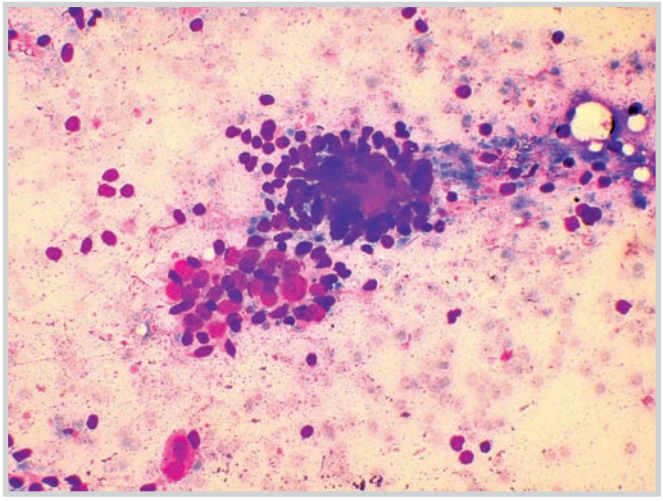

Fig. 33.24: Granulosa cell tumor: Cells are arranged around pinkish material (MGG × MP)

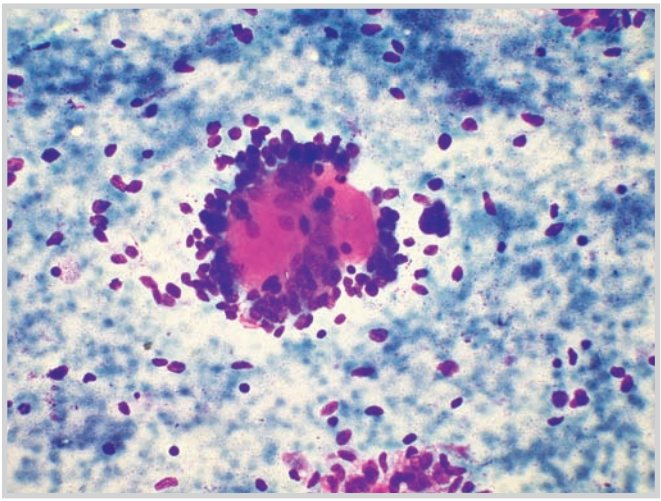

Fig. 33.25: Granulosa cell tumor: Higher magnification showing better morphology of the previous structure (MGG × HP)

Cytology

Fine needle aspiration cytology smears of the granulosa cell tumor show both discrete and small cohesive clusters of cells (**Box 33.9**). Occasionally, the cells are arranged around deep pink PAS positive material. The cells are round with scanty to moderate amount of pale cytoplasm with indistinct borders. Unlike adenocarcinoma, the nuclei of the cells are monomorphic, round and often have characteristic coffee bean like groove.

Sertoli Cell Tumor

Cytological diagnosis of sertoli cell tumor is difficult. The smear shows discrete monomorphic round to oval cells (**Figs 33.26** and **33.27**). Occasional tubules are also noted. The background may be fatty vacuolated.

BOX 33.9 Granulosa cell tumor

- Discrete cells
- Cells arranged around pink material
- Monomorphic nuclei
- Longitudinal coffee bean like nuclear groove

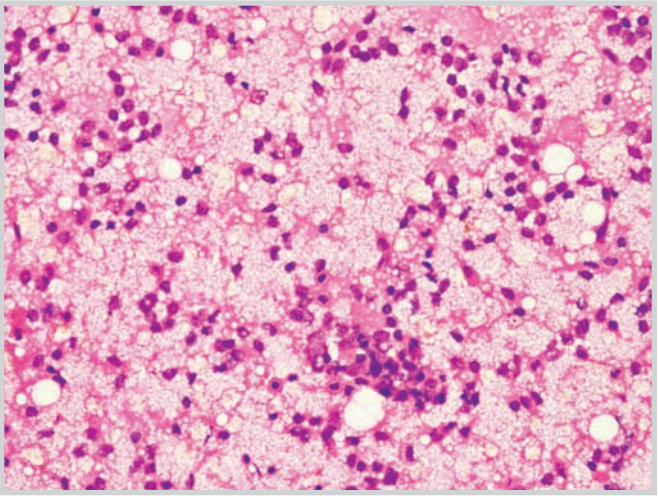

Fig. 33.26: Sertoli cell tumor: Monomorphic round to oval cells in vacuolated background (H & E × MP)

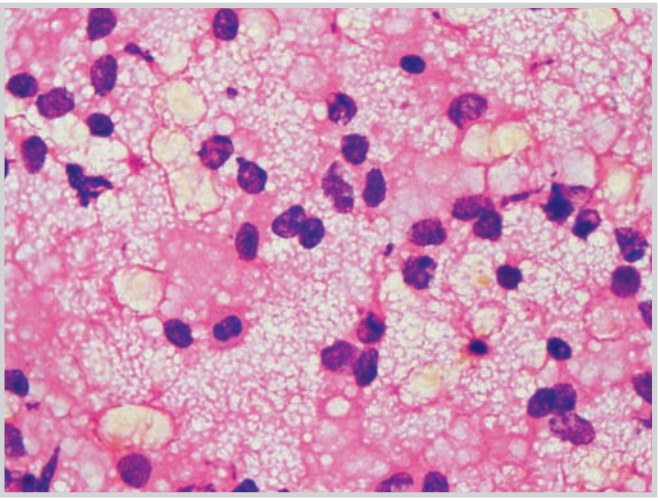

Fig. 33.27: Sertoli cell tumor: Higher magnification showing better morphology. Round cells with blunt nuclear chromatin (H & E × HP)

PROSTATE[20,21]

The major indication of FNAC of prostate is to differentiate from benign prostatic enlargement from malignancy. Prostate-specific antigen (PSA) is a useful serum biomarker for screening of prostate carcinoma. Core needle biopsy of the prostate along with serum PSA level estimation has greatly replaced FNAC of the prostate. However,FNAC has the added advantage of multiple sampling from the different area of the prostate. The detailed description of prostate FNAC has been described in previous chapter. FNAC of prostate is almost free of any complications except minor hematuria, pyrexia and hematospermia. Diagnostic accuracy of prostate FNAC is well established.

Normal Cells in Prostate FNAC

Prostate epithelial cells: The normal prostate epithelial cells are usually present in small monolayered sheets. The cells are arranged as honeycomb-like pattern. The individual cells are round, with scanty cytoplasm and round monomorphic nuclei having indistinct nucleoli.

Seminal vesicle cells: These cells are quite large with hyperchromatic enlarge nuclei. Seminal vesicle cells may be mistaken as malignant cells. However, these cells are readily identifiable because of yellow lipochrome pigment in the cytoplasm and accompanying spermatozoa.

Spermatozoa: Occasionally, spermatozoa are seen in prostate FNAC.

Rectal epithelial cells: Benign columnar epithelial cells from the rectum are also seen in FNAC smears of the prostate. The cells are columnar in shape with round monomorphic nuclei and vacuolated cytoplasm.

*Urothelial cells***:** The lining epithelial cells of the bladder may be seen if the needle passes through the bladder wall to the superficial mucosa.

Benign Prostatic Hyperplasia

Cytology (Fig. 33.28)

The cytology smear of the benign prostatic hyperplasia shows multiple clusters of monolayered sheets and occasionally gland like arrangement (**Box 33.10**). The cells are round with scanty cytoplasm and remarkably monomorphic nuclei with regular nuclear membrane. Nuclear chromatin is finely granular with indistinct nucleoli.

Carcinoma

Cytology (Figs 33.29 to 33.31)

Fine needle aspiration cytology smear of the prostate shows three-dimensional tight clusters of cells, multiple microacinar arrangement and dyscohesive cells (**Box 33.11**). There are abundant microacinar structures present in adenocarcinoma. Considerable amount of dissociated cells are also present. Nuclei of the cells are enlarged, pleomorphic with high nucleocytoplasmic (N/C) ratio. Nuclear margin is irregular with large prominent nucleoli. The most important characteristics of adenocarcinoma are three-dimensional clusters, microacini, dissociated cells and pleomorphic cells with high N/C ratio and prominent macronucleoli.

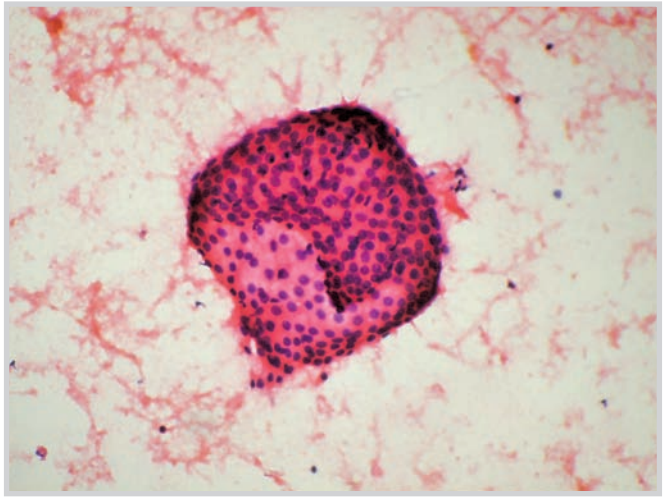

Fig. 33.28: Prostate epithelial cells: Monolayer sheet of benign prostatic epithelial cells (H & E × MP)

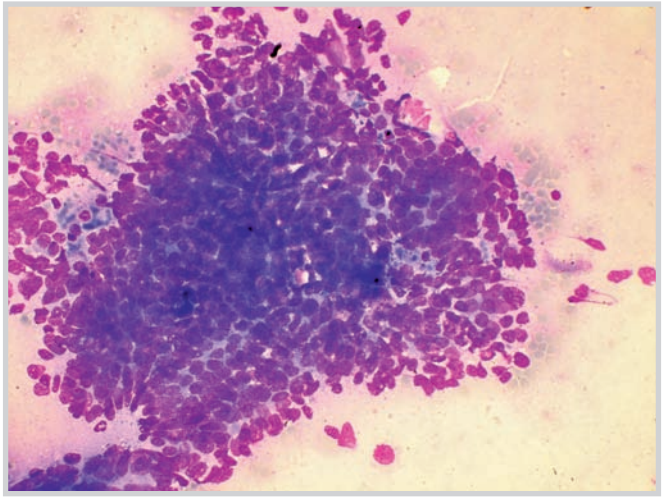

Fig. 33.30: Adenocarcinoma of prostate: Three-dimensional clusters of malignant cells (MGG × MP)

BOX 33.10 Benign prostatic hyperplasia

- Monolayered sheets
- Round, monomorphic nuclei
- Granular chromatin.
- No nuclear enlargement

BOX 33.11 Adenocarcinoma of prostate

- Three dimensional clusters, dissociated cells and multiple microacinar structures
- Nuclear enlargement and high nucleocytoplasmic (N/C) ratio
- Prominent nucleoli
- Irregular nuclear membrane.
- Prostate specific antigen (PSA) is high (more than 4 ng/ml)

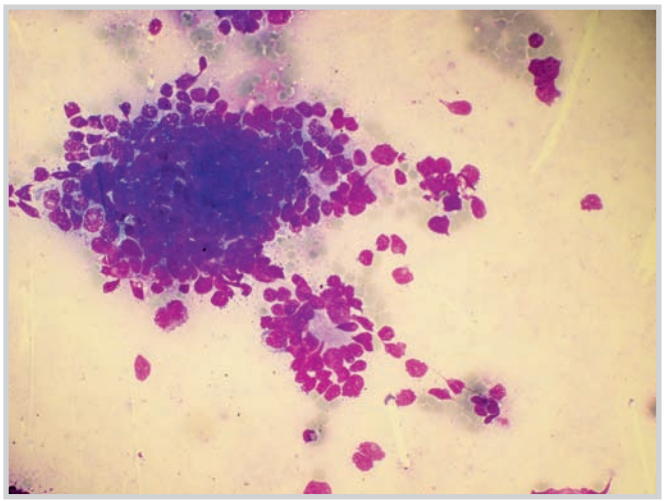

Fig. 33.29: Adenocarcinoma of prostate: Cluster and glandular arrangement of cells (MGG × MP)

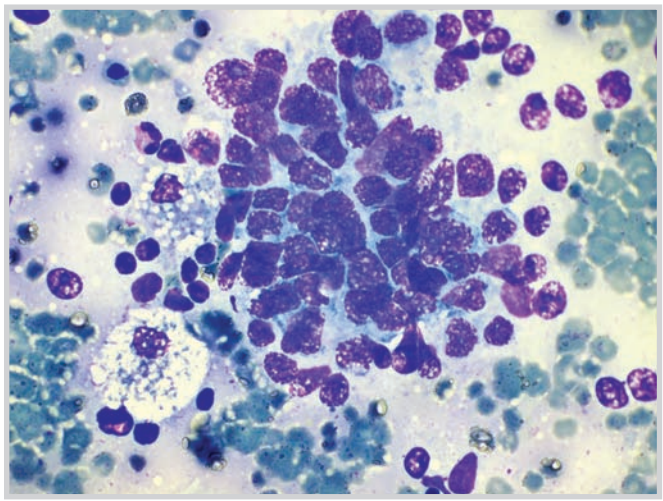

Fig. 33.31: Adenocarcinoma of prostate: Cells with moderately pleomorphic nuclei having fine chromatin and prominent nucleoli (MGG × HP)

Differential Diagnosis

- *Seminal vesicle cells*
- *Rectal mucosal cell:* Rectal mucosal cells may be misinterpreted as malignant cells. However, individual cells do not show any features of malignancy.
- *Well differentiated carcinoma versus benign lesion:* It is often difficult to differentiate well-differentiated carcinoma from the benign prostatic hyperplasia.

Other tumors: Tumors from the adjacent area such as rectum or bladder may also involve prostate.

Rarely, sarcoma may develop from the native prostatic stroma and muscle. *Leiomyosarcomas* are most frequently seen among all other sarcomas. The tumor cells are usually arranged in small tight clusters and fascicles. The cells are elongated with spindle-shaped blunt ended nuclei. The cells of leiomyosarcoma are positive for desmin and smooth muscle actin.

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CHAPTER 34

Soft Tissue Lesions

Chapter Contents

INTRODUCTION

Soft tissue tumor comprises of wide variety of lesions with different architectural patterns. It is often difficult to assess the exact histological type of soft tissue lesions on fine needle aspiration cytology (FNAC). The primary diagnosis of soft tissue tumor with the help of FNAC is not well-accepted and it is still a controversial issue. In fact, there is a negative attitude of surgeons, oncologist and even histopathologists regarding FNAC diagnosis of soft tissue lesions.[1-3] However, FNAC is useful in recurrent soft tissue sarcomas (STSs), metastatic sarcomas, and also to confirm various benign lesions (**Box 34.1**). It also provides necessary information about the nature of the soft tissue lesion. Ancillary investigations can be done from the FNAC material to reach a conclusive diagnosis in certain situations.[4]

Compared to open biopsy or core needle biopsy, FNAC of soft tissue can be done in outpatient department and the procedure is well-tolerated, rapid and cost effective. If needed, repeat samples can be taken for ancillary investigations such as immunohistochemistry, electron microscopy, deoxyribonucleic acid ploidy analysis and molecular genetics (**Box 34.2**).

Fine needle aspiration cytology of soft tissue lesion is almost free of any complication. Needle tract seeding is a myth and has no practical importance.

BOX 34.1 **Indications of FNAC of soft tissue lesions**

- Initial confirmation of nature of the lesion for further management
- Recurrent sarcoma
- Metastatic sarcoma
- To exclude a metastatic tumor in the soft tissue

BOX 34.2 **Advantages of FNAC of soft tissue lesions**

- Well-tolerated
- Rapid
- Cost effective
- Multiple sampling
- Ancillary investigation possible

DIAGNOSTIC ACCURACY

The overall diagnostic sensitivity and specificity of soft tissue tumors are more than 90%.[5-10] The false-negative rate of soft tissue tumor is as high as 15%. The main causes of false-negative

BOX 34.3 False-negative diagnosis

- Inadequate sampling
  - Less number of needle passes
  - Hyalinized tissue
  - Collagenous tissue
  - Necrosis
  - Richly vascular
  - Deep seated lesion may be missed
- Nonrepresentative sample
  - Sampling from adjacent reactive tissue
- Interpretation error

BOX 34.4 Fine needle aspiration cytology techniques

- Needle: 22 G
- Multiple areas should be sampled
- Air-dried and alcohol-fixed smear
- MGG and Papanicolaou's [or hematoxylin and eosin (H&E)] staining
- On-site assessment of material
- Material for cell block: Immunocytochemistry
- Material for other ancillary studies: flow cytometry, molecular genetics

diagnosis are inadequate sampling, nonrepresentative sampling and misinterpretation of the smear (**Box 34.3**). Inadequate or suboptimal sampling may be due to FNAC of richly vascular tissue such as hemangioma or angiosarcoma. The tumor may be hyalinized or collagenous and FNAC may yield poor amount of cells. Nonrepresentative sampling may be due to entry of the needle to the adjacent reactive tissue. The rate of false-negative report can be reduced if multiple sampling is done from the different areas of the tumor and immediate assessment of the material is done. False-positive diagnosis of FNAC is about 5%. In case of any doubt, histopathological examination of the tumor should be recommended. Mutilating surgery should not be done on the basis of primary FNAC diagnosis.

FNAC TECHNIQUE AND PREREQUISITES FOR INTERPRETATION OF SMEAR

Fine needle aspiration cytology of soft tissue lesion is done as usual with a syringe, pistol handle and needle (**Box 34.4**). The diameter of the needle should not be more than 0.7 mm (22 G). FNAC should be done from multiple sites to avoid the problem of tumor heterogeneity and also to get adequate representative tissue. Immediate rapid Giemsa staining may provide necessary information about the adequacy of the material and also about the need for ancillary studies. Both alcohol-fixed and air-dried smears should be kept for Papanicolaou's stain and May-Grünwald-Giemsa (MGG) staining, respectively. One part of the aspirate should be collected in 10% buffered formalin for cell block to do further immunostaining. If necessary, a repeat FNAC could be done for flow cytometry and molecular genetics study.

For accurate diagnosis of soft tissue tumor following information is needed:

- *Age:* Many soft tissue tumors are age specific. Rhabdomyosarcoma and soft tissue Ewing's tumor usually occur in young patients.
- *Chief complaints:* Rapidly growing tumor within a short duration is likely to be malignant.
- *Size:* Tumor with more than 5 cm diameter should be carefully interpreted as the chance of malignancy is more in large sized mass.
- *Computed tomography scan or magnetic resonance imaging:* Deeper tissue or bone infiltration by the tumor indicates its malignant behavior.

In addition, a past history of sarcoma or other malignancy is helpful in accurate interpretation of the FNAC smear.

Fine needle aspiration cytology diagnosis of soft tissue tumor can be given as benign, sarcoma, other malignancy and inadequate.[10] In case of difficulty, a thorough description of the smear could be given along with possible differential diagnosis[5,11,12]

Many authors recommend the use of cytomorphological subtypes of the soft tissue lesions based on predominant cell morphology such as:[13,14]

- *Spindle cell type*: Fibrosarcoma, leiomyosarcoma (LS), malignant peripheral nerve sheath tumor, synovial sarcoma (SS) (monophasic), dermatofibrosarcoma protuberans.
- *Round cell type:* Embryonal and alveolar rhabdomyosarcoma, soft tissue Ewing's tumor and round cell liposarcoma (LPS).
- *Pleomorphic cell type:* Malignant fibrous histiocytoma, pleomorphic LPS and pleomorphic rhabdomyosarcoma.
- *Biphasic tumors:* Synovial sarcoma.

However, there may be overlapping of the cell morphology and it may be difficult to put the lesion in one such category.

Exact histological subclassification of soft tissue tumor may not be always feasible. Grading of STS probably is more important than histological subtyping regarding management and prognosis. Currently, National Cancer Institute (NCI) grading system proposed by Costa et al. and the French grading system proposed by Guillou et al. from the Fédération Nationale des Centers de Lutte Contré le Cancer (FNCLCC) are commonly used in grading STS.[15,16] NCI system graded STS in three grades: grade 1, 2 and 3. This system uses grading of STS based on histological type, cellularity, pleomorphism, mitotic rate and necrosis. Depending on the amount of necrosis, the grade 2 (less than 15% necrosis) and grade 3 (more than 15% necrosis) STSs are assigned. FNCLCC system of grading gives importance more on tumor differentiation, mitotic rate and amount of tumor necrosis. A score is attributed to each factor and a combined score is used for grading of STS. We have successfully used NCI grading system to grade STS.[17] However, a two-tier grading system is also proposed on cytology smear for better reproducibility (**Table 34.1**).[18,19]

World Health Organization (WHO) classified soft tissue tumor according to histological type and behavior of the tumor (**Box 34.5**).[20]

TABLE 34.1: Two-tiered grade of sarcoma

Features	*Low-grade sarcoma*	*High-grade sarcoma*
Cellularity	Low	High
Nuclear atypia	Minimal	Moderate to severe
Nuclear overlap	Minimal	Moderate to marked
Necrosis	Absent	Present
Mitosis	Absent	Present

BOX 34.5 **WHO classification of soft tissue tumor (modified)**

- Tumors of adipocytes
  - Benign
  - Lipoma and it variants such as myolipoma, angiolipoma, spindle cell, pleomorphic lipoma
- Intermediate (locally aggressive)
  - Atypical lipomatous tumor/well-differentiated liposarcoma
- Malignant
  - Dedifferentiated liposarcoma, myxoid liposarcoma, round cell liposarcoma, pleomorphic liposarcoma, mixed-type liposarcoma, liposarcoma, not otherwise specified
- Fibroblastic/myofibroblastic tumors
  - Benign
  - Nodular fasciitis, proliferative fasciitis, proliferative myositis, myositis ossificans
- Intermediate (locally aggressive)
  - Superficial fibromatoses, desmoid-type fibromatoses, lipofibromatosis
- Intermediate (rarely metastasizing)
  - Solitary fibrous tumor, hemangiopericytoma, infantile fibrosarcoma
- Malignant
  - Adult fibrosarcoma, myxofibrosarcoma, low-grade fibromyxoid sarcoma
- Fibrohistiocytic tumors
  - Benign
  - Giant cell tumor of tendon sheath, diffuse-type giant cell tumor

Contd...

Contd...

- Intermediate (rarely metastasizing)
  - Plexiform fibrohistiocytic tumor, giant cell tumor of soft tissues
- Malignant
  - Pleomorphic malignant fibrous histiocytoma (MFH), giant cell MFH, inflammatory MFH
- Smooth muscle tumors
  - Leiomyoma
  - Leiomyosarcoma
- Pericytic (perivascular) tumors
  - Glomus tumor, malignant glomus tumor
- Skeletal muscle tumors
  - Benign
  - Rhabdomyoma
- Malignant
  - Embryonal rhabdomyosarcoma, alveolar rhabdomyosarcoma, pleomorphic rhabdomyosarcoma
- Vascular tumors
  - Benign
  - Hemangiomas, epithelioid hemangioma, lymphangioma
- Intermediate (locally aggressive)
  - Kaposiform hemangioendothelioma
- Intermediate (rarely metastasizing)
  - Retiform hemangioendothelioma, papillary intralymphatic angioendothelioma, Kaposi sarcoma
- Malignant
  - Epithelioid hemangioendothelioma, angiosarcoma of soft tissue
- Chondro-osseous tumors
  - Soft tissue chondroma, mesenchymal chondrosarcoma, extraskeletal osteosarcoma
- Tumors of uncertain differentiation
  - Benign
  - Myxoma
- Intermediate (rarely metastasizing)
  - Mixed tumor, Myoepithelioma
- Malignant
  - Synovial sarcoma, epithelioid sarcoma, alveolar soft part sarcoma, clear cell sarcoma of soft tissue, PNET/extraskeletal Ewing's tumor, desmoplastic small round cell tumor, neoplasms with perivascular epithelioid cell differentiation (PEComa)

LIPOMA AND ITS VARIANTS[7,8]

This is the most common soft tissue tumor and represents about 50% of all soft tissue tumors. The tumor is common in elderly obese people. Lipoma may occur in any anatomic locations. It may be seen in superficial subcutaneous tissue or deep soft tissue.

Cytology

Fine needle aspiration cytology smears shows multiple clusters of mature adipocytes (**Box 34.6**). The fat cells show abundant vacuolated cytoplasm with centrally placed monomorphic round nuclei. The cytoplasm is thin and peripherally pushed. Smears may also show fat filled histiocytes and thin capillaries (**Fig. 34.1**).

Spindle Cell Lipoma

They probably represent a single entity with overlapping clinical, morphologic, and cytogenetic features. Spindle cell lipoma or pleomorphic lipoma commonly occurs in posterior part of neck, upper back and shoulder area as well-circumscribed, painless, slow growing mass (**Figs 34.2** and **34.3**).

BOX 34.6 Lipoma

- Mature adipocytes
- Abundant vacuolated cytoplasm
- Central monomorphic bland nuclei

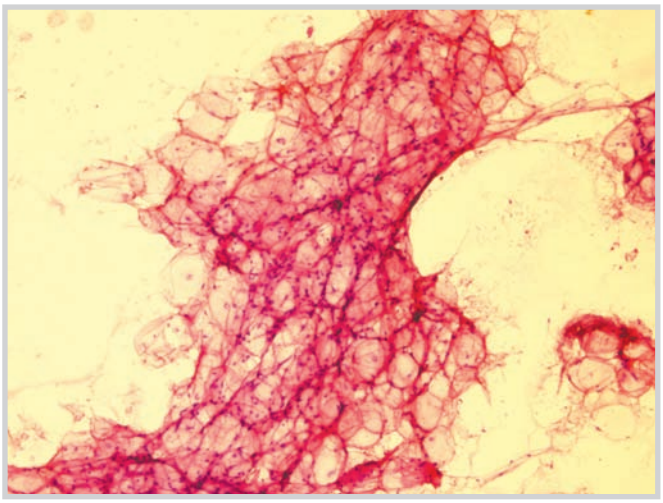


Fig. 34.1: Lipoma: lobules of mature fat [hematoxylin and eosin × medium power (MP)]

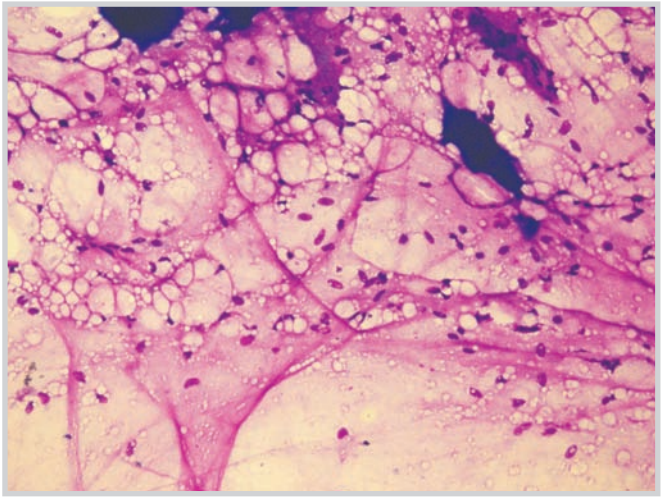


Fig. 34.2: Spindle cell lipoma: predominantly spindle cells (May-Grünwald-Giemsa × MP)

Cytology

Fine needle aspiration cytology smear shows a mixed population of spindle cells, adipocytes and collagen bundles (**Box 34.7**). The spindle cells are arranged in short fascicles in between the adipocytes. The spindle cells are usually bland; however, they may show mild nuclear pleomorphism. In addition, the smears may also show hyaline, ropy collagen fibers and mast cells.[21]

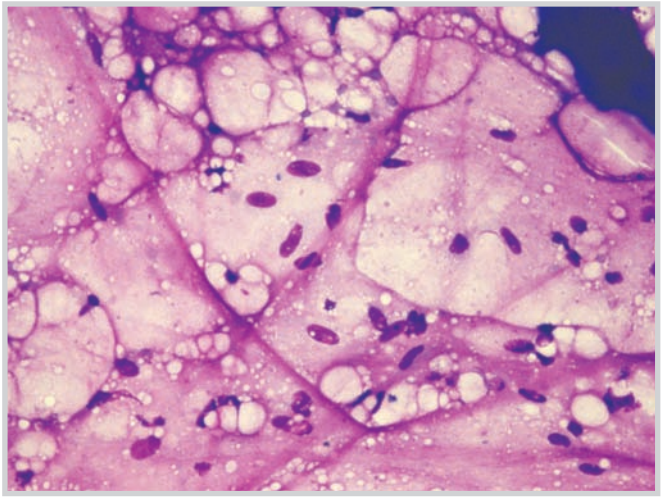


Fig. 34.3: Spindle cell lipoma: higher magnification showing better morphology of the spindle cells [May-Grünwald-Giemsa × high power (HP)]

BOX 34.7 Spindle cell lipoma

- Adipocytes
- Spindle cells
  - Bland looking nuclei
  - Small fascicles
- Collagen bundles
- Mast cells
- No lipoblasts

Pleomorphic Lipoma

Fine needle aspiration cytology smear shows fat cells with enlarged, moderately pleomorphic, hyperchromatic nuclei and floret cells. Floret like cells may also be noted.

The floret cells show large round cells with abundant cytoplasm and multiple nuclei arranged in a circle or semicircle manner (**Box 34.8**). No lipoblasts are seen.

Differential Diagnosis

Liposarcoma: Pleomorphic lipoma and atypical lipoma may be mistaken for LPS.

Hibernoma

This is a rare benign lipomatous tumor composed of brown fat cells. It commonly occurs in young adults and the mean age of the tumor is 38 years. The common sites of hibernoma are thigh, trunk, upper extremity and neck region.

Cytology

Fine needle aspiration cytology smears show three populations of cells: (1) mature adipocytes, (2) hibernoma cells and (3)

BOX 34.8 Pleomorphic lipoma

- Adipocytes with enlarged hyperchromatic nuclei
- Floret cells
  - Multiple nuclei arranged in semicircular manner
- No lipoblasts

lipoblast like cells. Hibernoma cells are large with pale multiple small uniformly vacuolated cytoplasm. Nuclei are round centrally placed. The lipoblast like cells are large with larger fat vacuoles. In addition, the smear also shows delicate thin capillaries.[22]

LIPOSARCOMAS[23-26]

Atypical Lipomatous Tumor/Well-differentiated Liposarcoma (Figs 34.4 to 34.7)

The atypical lipomatous tumor (ALT) is considered as an intermediate grade of malignant tumor and is synonymous with well-differentiated LPS. ALT represents 40–45% of all LPSs and commonly occurs in the sixth decade of life. The common anatomical locations of the tumor are deep soft tissue of the extremities, retroperitoneum, the paratesticular area and the mediastinum.

Cytology

Fine needle aspiration cytology smears show fat cells with enlarged pleomorphic nuclei. The nuclei may be multilobated. There may be admixture of lipoblasts. The presence of lipoblasts in a sarcoma usually indicates LPS. The important characteristics of lipoblasts are:

- Multivacuolated round cells
- Scalloping vacuoles around nucleus
- Occasionally compressed nuclei may give signet ring-like appearance

It is often difficult to distinguish such lesion from lipoma. It is, therefore, always advisable to have histopathological examination in any deep lipomatous lesion.[23]

Myxoid Liposarcoma

Myxoid liposarcoma (MLS or myxoid LPS) is the second most common type of LPS and represents about 10% of all adult STSs. MLS is commonly seen in deep soft tissue of thighs. The tumor commonly occurs in the fourth and fifth decades of life. The

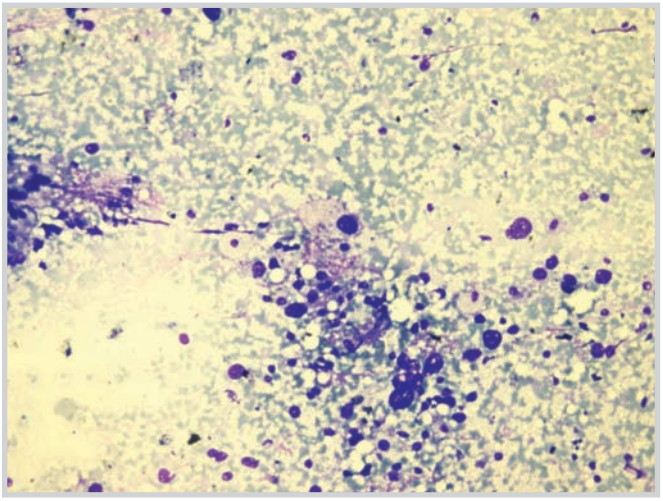


Fig. 34.5: Well-differentiated liposarcoma: scattered large atypical cells with abundant vacuolated cytoplasm (May-Grünwald-Giemsa × MP)

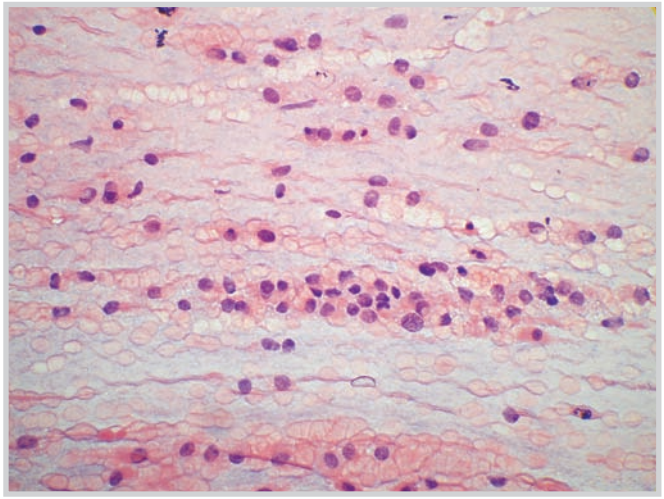


Fig. 34.4: Well-differentiated liposarcoma: monomorphic round cells with moderate amount of vacuolated cytoplasm (hematoxylin and eosin × MP)

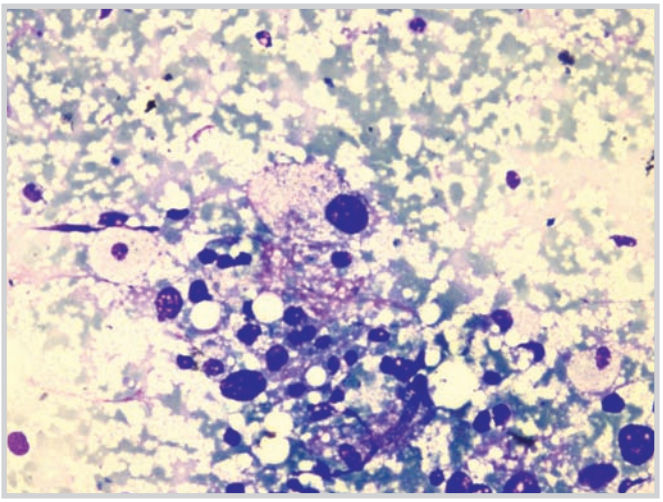


Fig. 34.6: Well-differentiated liposarcoma: scattered cells with enlarged and pleomorphic nuclei (May-Grünwald-Giemsa × HP)

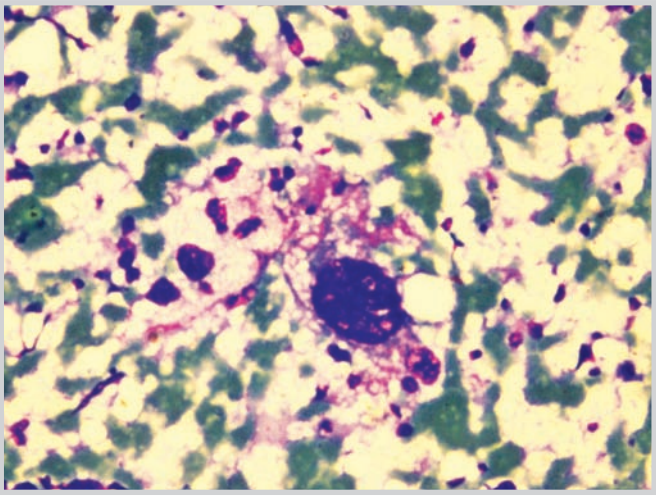


Fig. 34.7: Well-differentiated liposarcoma: higher magnification showing better morphology of the atypical cells [May-Grünwald-Giemsa × oil immersion (OI)]

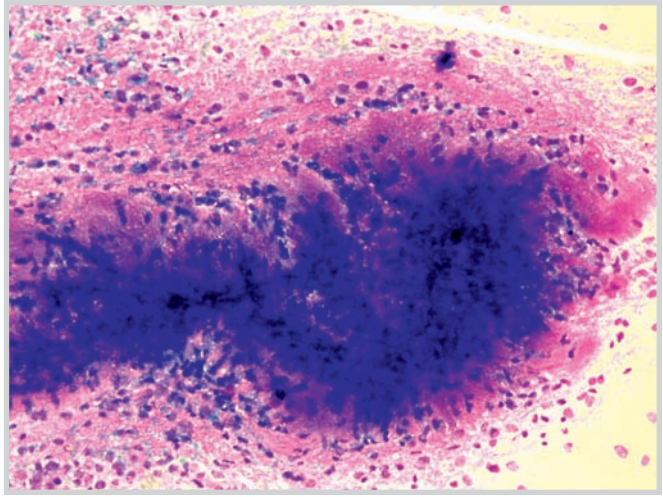


Fig. 34.8: Myxoid liposarcoma: thin arborizing capillaries in myxoid stroma (May-Grünwald-Giemsa × MP)

BOX 34.9 Myxoid liposarcoma

- Abundant granular myxoid material
- Thin-walled arborizing capillaries
- Uni or multivacuolated lipoblasts
- Round cells with high N/C ratio
- Spindle-shaped cells with cytoplasmic vacuoles

patient presents with a large painless mass within the deep soft tissue.

Cytology

Fine needle aspiration cytology smear shows tumor cells entrapped in the abundant granular myxoid material. There are many arborizing thin walled capillaries (**Box 34.9**). The tumor cells show oval shaped bland nuclei with high nucleocytoplasmic ratio (N/C ratio). The presence of univacuolated or multivacuolated lipoblasts with scalloped nuclear margin is essential for diagnosis of myxoid LPS. This tumor lacks nuclear pleomorphism, bizarre tumor giant cells or any mitotic activity (**Figs 34.8** to **34.10**).

Pleomorphic Liposarcoma

Pleomorphic liposarcoma (PLS) is a high-grade sarcoma and accounts for 5% of all LPS. This tumor occurs in elderly patient and has no sex predilection. The most common location of this tumor is in the deep tissue of thighs. The patient usually presents as firm rapidly enlarging mass in the extremity.

Cytology

Fine needle aspiration cytology smears are usually rich in cells. The tumor cells are in dissociation and small loose clusters.

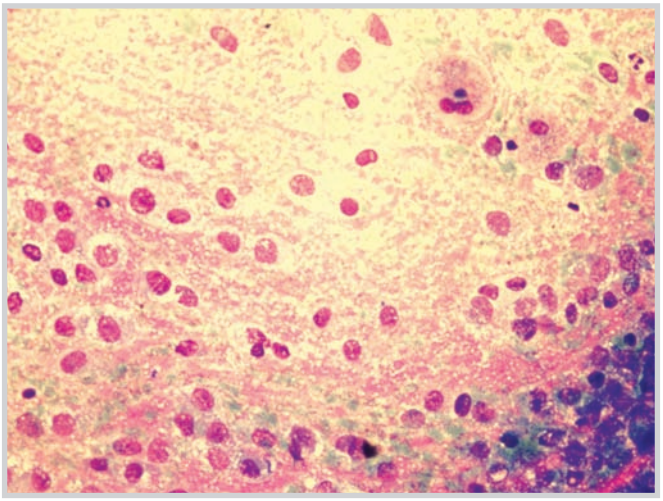


Fig. 34. 9: Myxoid liposarcoma: scattered round cells with vacuolated cytoplasm in the myxoid material (May-Grünwald-Giemsa × MP)

The cells show moderate amount of vacuolated cytoplasm. The nuclei are markedly pleomorphic with coarse chromatin and prominent nucleoli. Many multinucleated giant cells and bizarre mononuclear cells are also noted (**Box 34.10**). The smears also show variable number of atypical lipoblasts. The diagnosis of pleomorphic LPS rests on the identification of lipoblasts (**Figs 34.11** and **34.12**).[27]

Differential Diagnosis

Myxoid tumor versus MLS: Myxoid LPS may pose diagnostic difficulty with other soft tissue tumor with myxoid changes such as intramuscular myxoma (IM) or myxoid variant of malignant fibrous histiocytoma. Intramuscular myxoma shows lack of branching capillaries and typical lipoblasts. Myxoid variant of malignant fibrous histiocytoma shows significant nuclear pleomorphism.

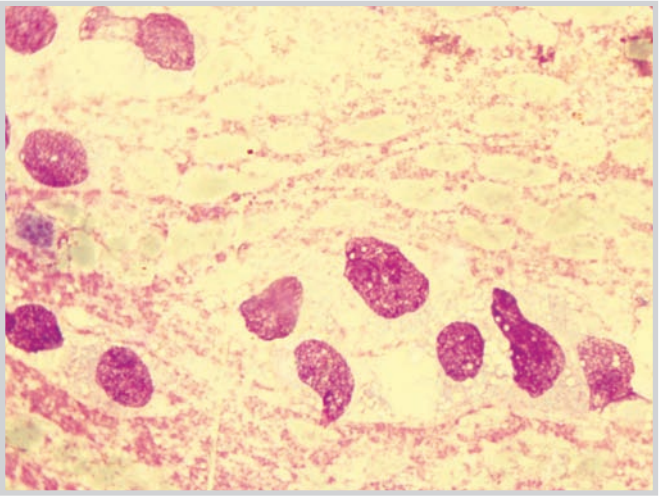


Fig. 34.10: Myxoid liposarcoma: higher magnification showing better morphology of the individual cells (May-Grünwald-Giemsa × OI)

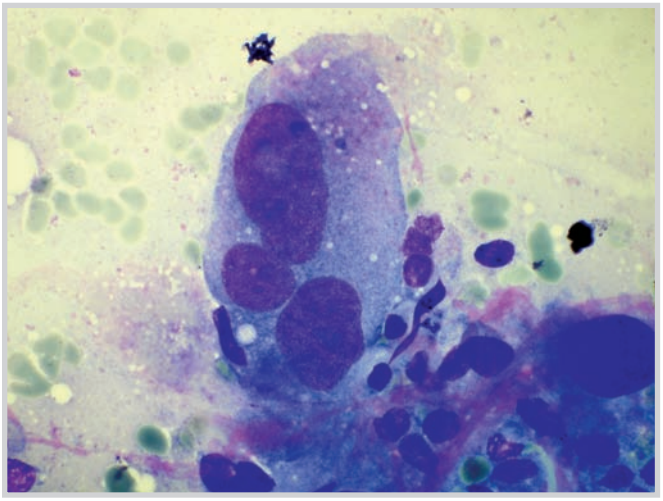


Fig. 34.12: Pleomorphic liposarcoma: higher magnification of the large bizarre cells (May-Grünwald-Giemsa × OI)

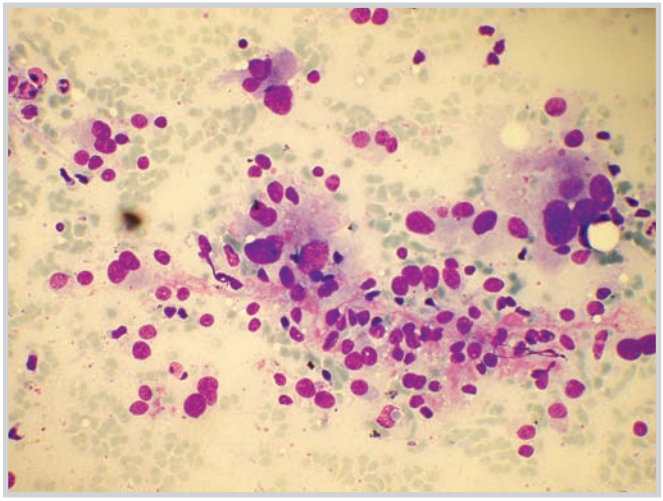


Fig. 34.11: Pleomorphic liposarcoma: large cells with markedly pleomorphic nuclei having moderate to abundant cytoplasm (May-Grünwald-Giemsa × MP)

BOX 34.10 Pleomorphic liposarcoma

- Large cells with vacuolated cytoplasm
- Markedly pleomorphic nuclei
- Coarse chromatin
- Prominent nucleoli
- Atypical lipoblasts
- Mitotic activities
- Necrosis

- *Other pleomorphic sarcomas versus PLS:* Other pleomorphic sarcomas such as malignant fibrous histiocytoma or rhabdomyosarcoma also come in the differential diagnosis of pleomorphic LPS.

FIBROBLASTIC/MYOFIBROBLASTIC LESION

Fibromatosis

Fibromatosis is a locally aggressive infiltrative tumor of fibroblasts that never metastasize. The exact etiology of this tumor is unknown. These tumors are composed of fibrous tissue and are broadly divided into two groups: (1) superficial and (2) deep. The superficial fibromatosis involve palmer, planter and penile region. The deeper fibromatosis include the abdominal and extra-abdominal region.

Cytology

Cytology smear shows scanty to moderate cellularity. The cells are in clusters and embedded in collagenous stroma (**Box 34.11**). The individual cells are oval to spindle-shaped with elongated nuclei and pointed ends. The nuclei are hyperchromatic with minimal atypia. The mitotic activity is absent. The background of the smear is clean and shows fragments of dense collagenous stroma.

Differential Diagnosis

Fibrosarcoma: Fibrosarcoma may be difficult to differentiate from fibromatosis. Absence of nuclear pleomorphism and mitotic activity favor a diagnosis of fibromatosis.

Nodular Fasciitis

Nodular fasciitis is a mass forming fibrous proliferation. It occurs in all age groups, but more commonly occurs in younger patients. Nodular fasciitis may affect any part of the body but it commonly involves the upper extremity, trunk and head. It grows rapidly and may simulate malignancy.

BOX 34.11 Fibromatosis

- Variable amount of cells
- Oval to spindle cell
- Spindle-shaped nuclei
- Bland chromatin
- Collagenous stroma
- No mitotic activity

BOX 34.12 Myositis ossificans

- Granular matrix material
- Myofibroblast
  - Oval to polygonal cells
  - Feathery cytoplasm
  - Eccentrically placed round nuclei
- Osteoblast
- Osteoclast

Cytology

Cytology smear shows cohesive clusters and dissociated cells in a myxoid background. The cells are arranged around thin capillaries. The individual cells have plump spindly nucleus with blunt ends. Nuclear chromatin is open with small nucleoli. Mitotic activity may be high; however, no atypical mitosis is seen.

Differential Diagnosis

Sarcoma: Increased cellularity and high mitotic activity may mislead the diagnosis of sarcoma.

Myositis Ossificans[28,29]

Myositis ossificans (MO) is a self-limiting reparative lesion. It characteristically occurs in a young male who may or may not recall the history of trauma. The swelling may occur anywhere in the body but commonly occurs in extremities, trunk, head and neck. The swelling shows the characteristic radiological appearance in its osteoid producing phase and may mislead the clinical diagnosis of perosteal osteosarcoma.

Cytology

Fine needle aspiration cytology of MO yields low cellularity. The aspiration may be hindered due to ossified material. The smear often contains fragment of muscles and that may raise the doubt about adequacy of the sample (**Box 34.12**). The cells are dispersed in dirty granular or metachromatic stromal material. Cytology smears of MO show many multinucleated osteoclastic giant cells, osteoblasts, proliferating fibroblasts and myofibroblasts embedded at places in pink stroma. The myofibroblasts are large ovoid or polygonal cells with abundant feathery cytoplasm and eccentrically placed round nuclei. The osteoblasts are round to oval with eccentric nuclei. The nuclei may show mild reactive atypia. The atypical osteoblasts are the potential source of misdiagnosis of this lesion as osteosarcoma (**Figs 34.13** to **34.15**).

Differential Diagnosis

Osteosarcoma: MO may be misdiagnosed as osteosarcoma due to the presence of atypical osteoblasts, large atypical myofibroblasts and clinical history of rapid growth. A careful observation of the nuclear character of osteoblasts and identification of myofibroblasts may help in the accurate diagnosis of MO.

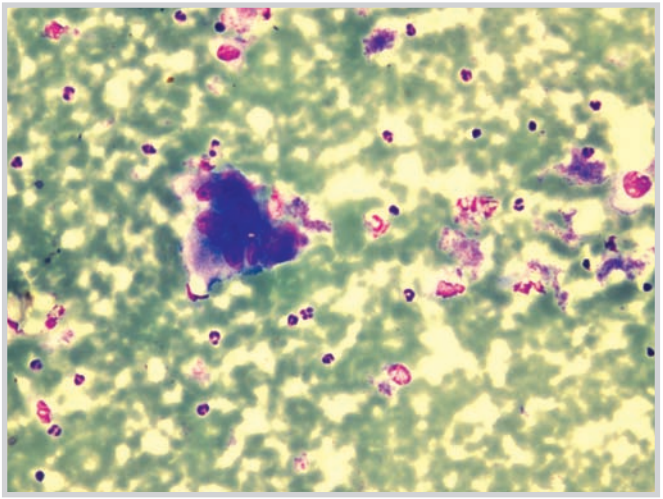


Fig. 34.13: Myositis ossificans: osteoclastic giant cells and occasional osteoblasts (May-Grünwald-Giemsa × MP)

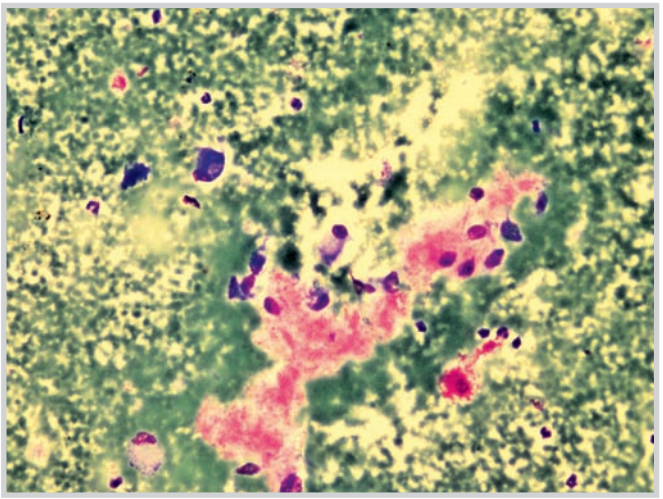


Fig. 34.14: Myositis ossificans: many osteoblasts and fragments of osteoid material (May-Grünwald-Giemsa × MP)

Fibrosarcoma[30]

Fibrosarcoma is a malignant tumor of fibroblastic origin (**Box 34.13**). The exact incidence of fibrosarcoma is difficult to assess as it is a tumor of exclusion. Possibly, fibrosarcoma represents 1–3% of all adult sarcomas.[31] It commonly occurs between

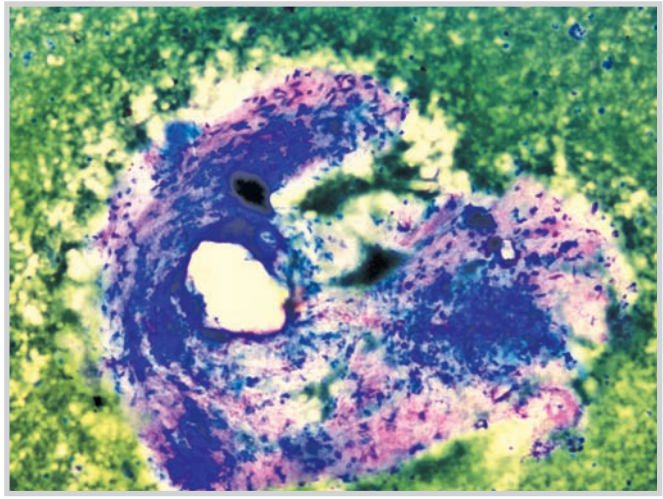


Fig. 34.15: Myositis ossificans: bundle of oval to spindle-shaped muscle cells and calcified material (May-Grünwald-Giemsa × MP)

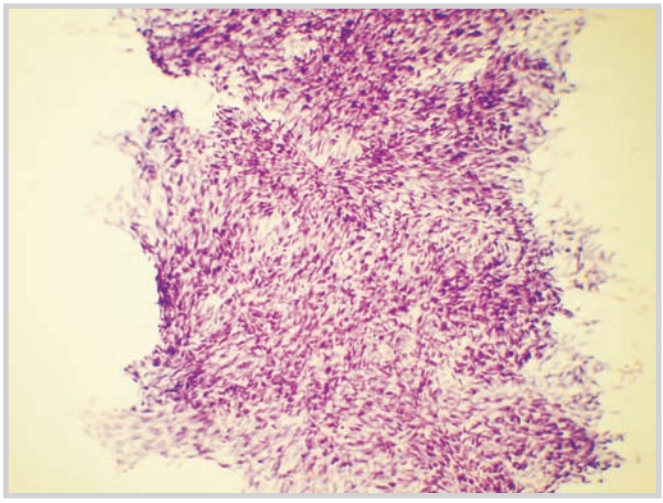


Fig. 34.16: Fibrosarcoma: large fascicle of spindle cells (hematoxylin and eosin × MP)

BOX 34.13 Fibrosarcoma

- Small fascicles of cells
- Herring bone pattern
- Spindle cells
- Pointed nuclei
- Irregular nuclear membrane
- Finely granular chromatin
- Variable nuclear pleomorphism
- Blood and necrosis

35 years and 55 years of age. It commonly affects the extremities, trunk, head and neck region. No specific predisposition factors of this tumor have been described, however, it may be associated with long term radiation exposure in course of therapy or foreign body implants in the body.[32] A distinct variant is the infantile fibrosarcoma that affects children.

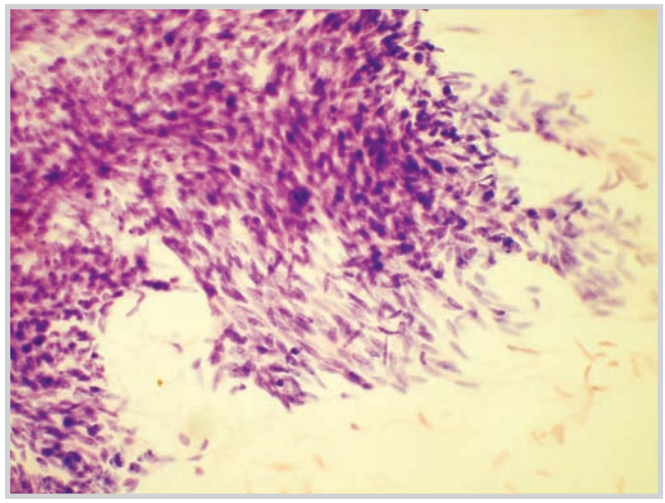


Fig. 34.17: Fibrosarcoma: spindle three-dimensional tight cluster of spindle cells (hematoxylin and eosin × HP)

Cytology

Fine needle aspiration cytology smears show abundant cellularity. The cells are arranged in small clusters, bundles and dissociated spindle cells. The cells are in interwoven pattern. Typical herring bone pattern may not be evident in case of cytology smear of fibrosarcoma. The tumor cells show elongated spindle-shaped nuclei with pointed ends and irregular nuclear membrane. The nuclear chromatin is finely granular with absent nucleoli. The cells show variable nuclear pleomorphism depending on the grade of the tumor. Nuclear pleomorphism is less in well-differentiated fibrosarcoma than in poorly differentiated fibrosarcoma. The amount of stromal matrix material varies and it is generally indirectly proportional to the cellularity of the smear. There may be blood and necrotic material in FNAC smear and the cells may be obscured. The diagnosis of fibrosarcoma is made by exclusion of other spindle cell sarcomas and the tumor is often missed in cytology smear (**Figs 34.16** to **34.18**).

Differential Diagnosis

Other spindle cell sarcomas: The differential diagnosis of fibrosarcoma comes along with other sarcomas of spindle cell morphology such as LS, SS, malignant peripheral nerve sheath tumor, etc.

Fibrohistiocytic Tumors

Giant Cell Tumor of Tendon Sheath[33,34]

Giant cell tumor of tendon sheath (GCTTS) is a benign lesion that occurs due to proliferation of synovial-like cells of the joints, bursae and tendon sheath. The tumor is also known as tenosynovitis. GCTTS may occur in any age group; however, it is commonly seen in 30–50 years of age. It commonly presents as a soft tissue nodule in relation to the tendon sheath of the fingers,

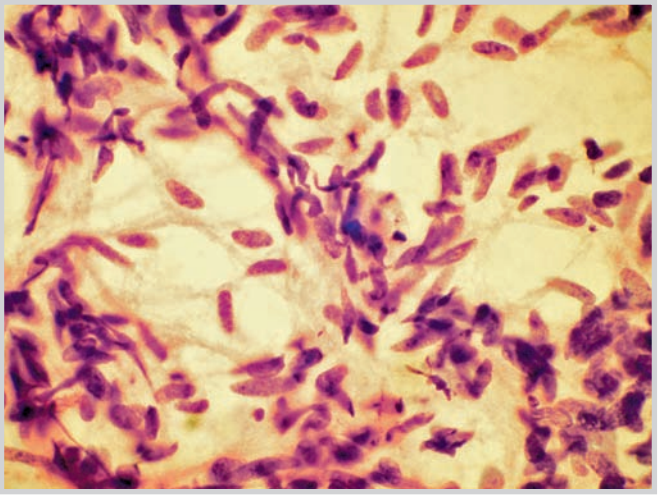


Fig. 34.18: Fibrosarcoma: higher magnification showing better morphology of the spindle cells. Note the elongated nuclei, homogenous chromatin and inconspicuous nucleoli (hematoxylin and eosin × OI)

wrist, ankle and knee. Radiological examination of the swelling always reveals a soft tissue mass free from the underlying bone.

Cytology

Fine needle aspiration cytology smear shows moderate to abundant cellularity. There are homogenous admixture of large number of multinucleated giant cells and mononuclear stromal cells (**Box 34.14**). The giant cells are morphologically identical with osteoclastic giant cells. The giant cells are large with widely variable size. They have average 20 nuclei. The nuclei often show grooves and indentation. Nuclear chromatin is stippled. Occasional prominent nucleoli are also seen. Nuclear character of these giant cells is almost similar to that of stromal cells.

The stromal cells are oval to polygonal with abundant cytoplasm and centrally placed variable shaped nuclei. Nuclear chromatin is finely granular. Nuclear grooves and occasional intranuclear inclusions are also seen. These cells show minimal nuclear pleomorphism and anisocytosis. Occasional mitotic figures may also be seen; however, no atypical mitosis is noted. The background of the smear often shows many hemosiderin laden macrophages (**Figs 34.19** to **34.21**).

Immunocytochemistry: Mononuclear stromal cells are positive for CD 68 and smooth muscle actin (SMA). Multinucleated giant cells are positive for CD68 and CD45. These cells also show tartrate resistant acid phosphatase.

Differential Diagnosis

Giant cell tumor of bone: Fine needle aspiration cytology smears of giant cell tumor of bone and GCTTS show almost similar cytological features. Giant cell tumor of bone shows characteristic bone involvement.

BOX 34.14 Giant cell tumor of tendon sheath

- Many multinucleated osteoclast like giant cells
- Mononuclear stromal cells
  - Oval to polygonal
  - Abundant cytoplasm
  - Round nuclei
  - Nuclear grooves
  - Intranuclear inclusions
- No atypical mitosis
- Hemosiderin laden macrophages

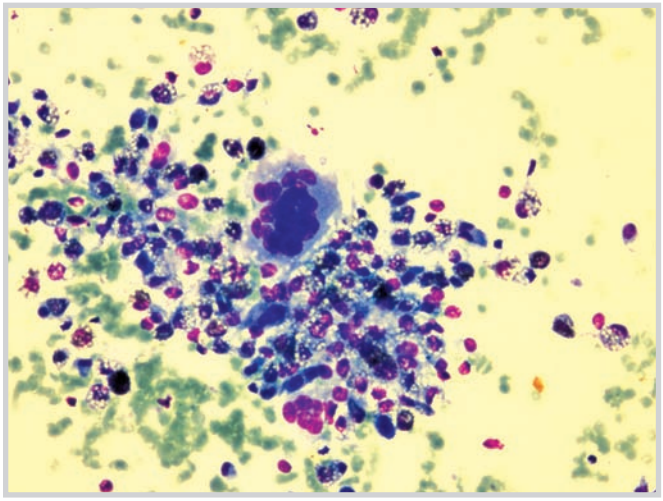


Fig. 34.19: Giant cell tumor of tendon sheath: multinucleated osteoclast like giant cells and pigment laden foamy histiocytes (May-Grünwald-Giemsa × MP)

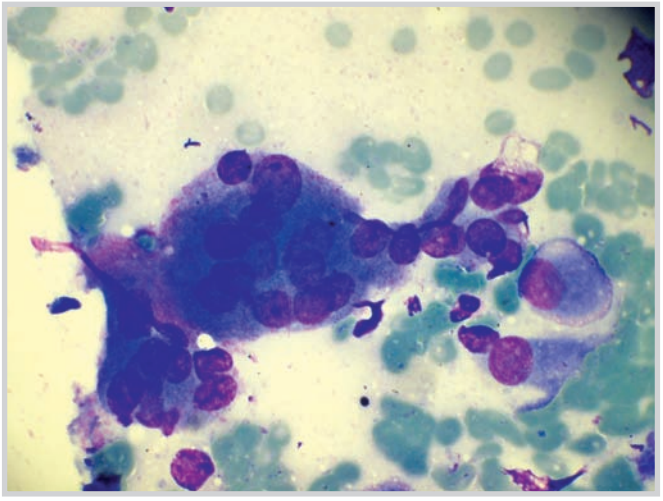


Fig. 34.20: Giant cell tumor of tendon sheath: multinucletaed osteoclast-like giant cells (May-Grünwald-Giemsa × HP)

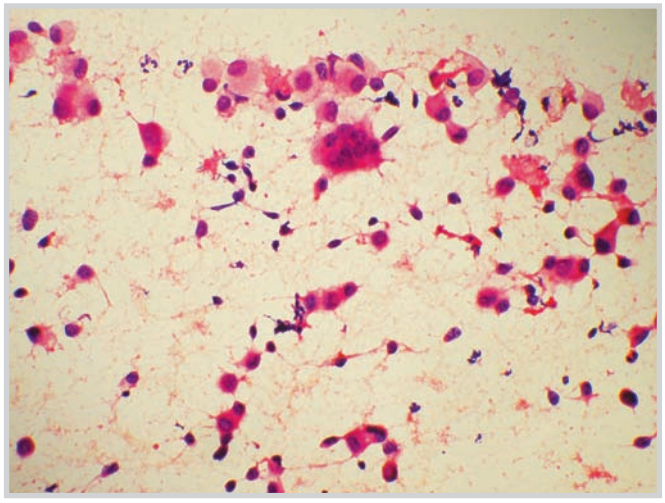


Fig. 34.21: Giant cell tumor of tendon sheath: histiocytes contain moderate amount of cytoplasm and monomorphic bland nuclei (hematoxylin and eosin × HP)

Malignant Fibrous Histiocytoma

This category of tumor is almost nonexistent. In fact, the concept of fibrohistiocytic differentiation has been challenged. The various histopathological types of MFH are nothing but the features of other sarcomas.[20] Therefore, the term "malignant fibrous histiocytoma (MFH)" is used only in a small subset of tumor that lacks definite differentiation.

Pleomorphic Malignant Fibrous Histiocytoma

This is a group of undifferentiated pleomorphic sarcoma. Originally, it was considered as the most common sarcoma. However, now it is demonstrated that wide variety of poorly-differentiated malignant neoplasm may demonstrate similar morphological features.[35]

Cytology

Fine needle aspiration cytology smear shows multiple clusters and dissociated spindle cells. The cells are often arranged in small fascicles. The tumor cells have moderate amount of pale blue vacuolated cytoplasm. The nuclei are plump to spindle-shaped with irregular coarse chromatin and prominent nucleoli. Many large pleomorphic bizarre tumor cells and giant cells are also seen.

Diagnostic Difficulties

- *Other pleomorphic sarcomas:* All the other pleomorphic sarcomas such as PLS and rhabdomyosarcoma should be excluded before diagnosis of this tumor.
- *Anaplastic carcinoma*: Any poorly differentiated carcinoma may simulate pleomorphic MFH.
- *Anaplastic large cell lymphoma*: Anaplastic large cell lymphoma (ALCL) lacks connective tissue matrix material and the cells of ALCL are more discrete. The cells of ALCL are positive for CD30 and activin receptor-like kinase-1 (ALK1).

TUMORS OF NERVE SHEATH

Benign Peripheral Nerve Sheath Tumor[36]

Schwannoma

Schwannoma arises from the Schwann cells of the nerve sheath. This tumor commonly affects the adults and usually involves flexor surface of the extremities, neck, mediastinum, retroperitoneum and cerebellopontine angle. Most of the tumors occur sporadically and occasional cases occur with neurofibromatosis type 2.

Cytology

Aspiration of schwannomas is often painful. FNAC smears show many clusters and discrete spindle cells. The cells are often embedded in pinkish fibrillary matrix material (**Box 34.15**). The tumor cells are spindle-shaped with long slender nuclei having pointed ends. The nuclei often show wavy margin and frequent kinking. Nuclear palisading like arrangement is also seen. At times the ancient schwannomas may show significant nuclear pleomorphism. These large pleomorphic cells may be falsely recognized as malignant cells. Mitotic activity is rarely seen in schwannomas. FNAC of ganglioneuroma shows admixture of ganglion cells along with many clusters of elongated spindle cells (**Figs 34.22** and **34.23**).

Differential Diagnosis

- *Sarcoma:* Ancient schwannoma may deceive as sarcoma because of nuclear pleomorphism.

Malignant Peripheral Nerve Sheath Tumor[37]

Malignant peripheral nerve sheath tumor (MPNST) is also known as *neurofibrosarcoma*. Half of the tumor arises as de novo and the other half arises from neurofibromatosis. The tumor develops from the large peripheral nerve or preexisting neurofibroma. This tumor commonly affects the proximal part of the extremities, pelvis and retroperitoneum. It is commonly seen in the third and fourth decades of life.

Cytology

Fine needle aspiration cytology smear shows abundant cellularity. The cells are arranged in small bundles and discretely.

BOX 34.15 Schwannoma

- Clusters and dissociated cells
- Palisading arrangement of cells
- Spindle cells
- Long slender nuclei
- Wavy nuclear margin with kinking
- Pointed nuclear ends

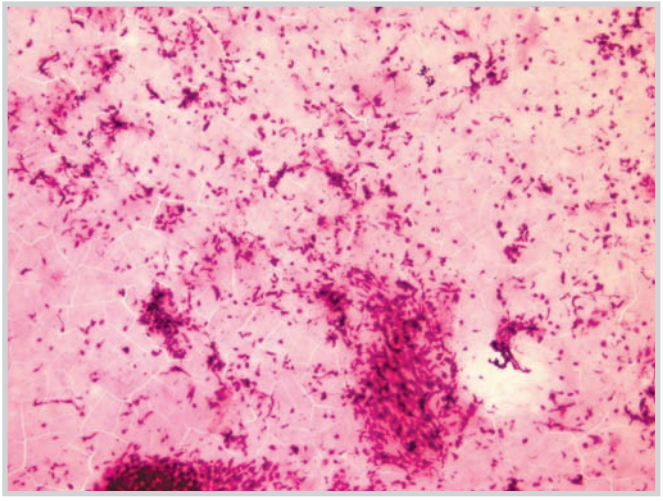


Fig. 34.22: Peripheral nerve sheath tumor: small clusters and discrete oval to spindle cells (hematoxylin and eosin × MP)

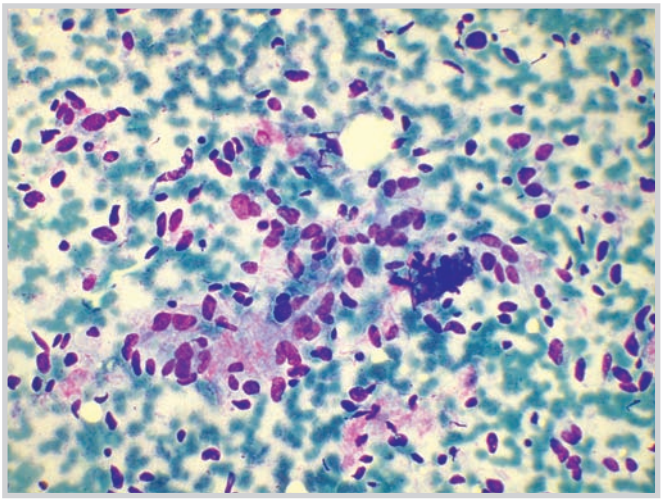


Fig. 34.24: Malignant peripheral nerve sheath tumor: discrete and loose cluster of spindle cells (May-Grünwald-Giemsa × HP)

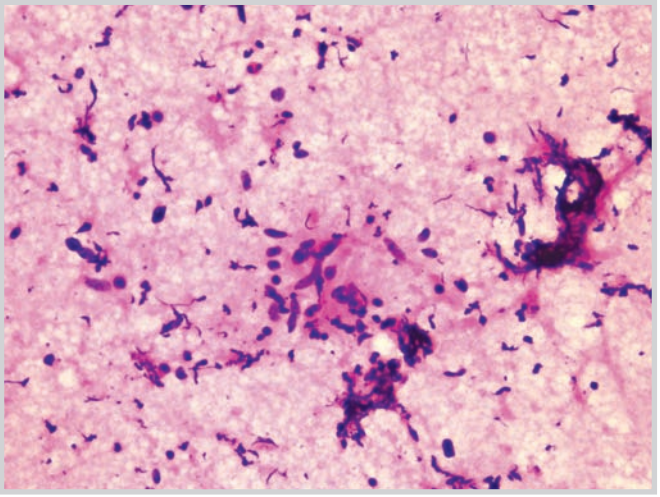


Fig. 34.23: Peripheral nerve sheath tumor: elongated spindle cells with pointed ends (hematoxylin and eosin × HP)

BOX 34.16 Malignant peripheral nerve sheath tumor

- Small fascicles
- Discrete cells
- Spindle-shaped cells
- Fibrillar cytoplasm
- Wavy kinked nuclei
- Pointed ends
- Fibrillar collagenous matrix

The tumor cells are embedded in pale violet fibrillary matrix (**Box 34.16**). The cells are elongated with delicate indistinct fibrillar cytoplasm. The nuclei are spindle-shaped with indentation and kinking and pointed ends. The nuclei show moderate pleomorphism with nuclear hyperchromasia and prominent nucleoli. N/C ratio is high. Mitotic activities are frequent with background necrosis (**Figs 34.24** and **34.25**).

Differential Diagnosis

- *Other spindle cell sarcoma:* Various spindle cell sarcomas such as fibrosarcoma, LSs and SS should be distinguished from MPNST.

TUMORS OF MUSCLE ORIGIN

Rhabdomyoma

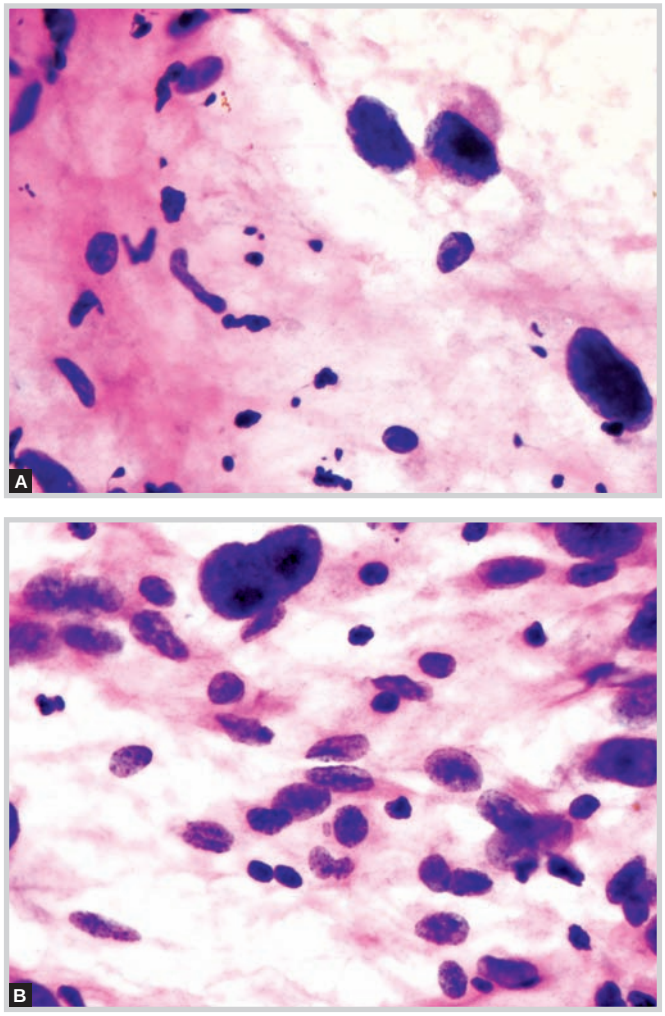
Rhabdomyoma (RM) is a benign soft tissue tumor with skeletal muscle differentiation. This tumor may occur both in adults and in children. About 90% of RM occurs in the head and neck region.

Cytology

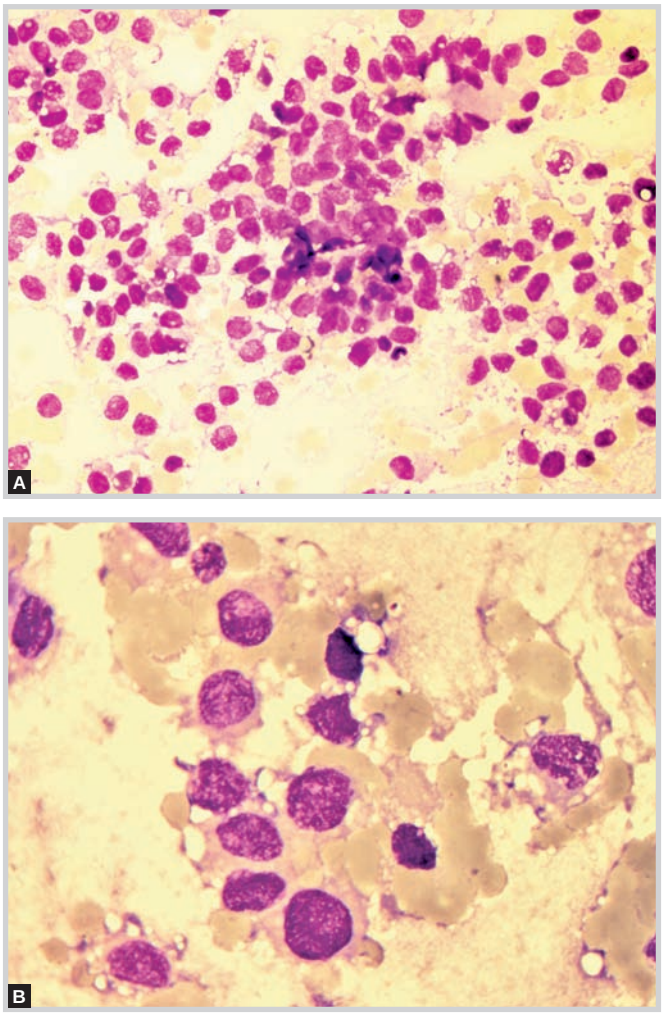
Fine needle aspiration cytology smears show large regenerating muscle fibers. The cells show abundant dense cytoplasm. Cytoplasmic cross striations are also evident. Nuclei are central and round shaped. Intranuclear cytoplasmic inclusions are often seen.

Rhabdomyosarcoma[38-40]

Rabdomyosarcoma (RMS) commonly occurs in children. It usually involves the head and neck region. RMS also occurs in the pelvic region particularly in bladder. This is the most common bladder tumor in children. There are three types of rhabdomyosarcoma: (1) pleomorphic, (2) embryonal and (3) alveolar. Out of these varieties pleomorphic RMS is the rarest. Rhabdomyoblasts are seen in all these three types in varying amounts (**Figs 34.26** and **34.27**). Rhabdomyoblasts may be divided into early, intermediate and late rhabdomyoblasts depending on the differentiation (**Table 34.2**).



Figs 34.25A and B: (A) Malignant peripheral nerve sheath tumor: spindle cells showing moderate to marked nuclear pleomorphism; (B) Malignant peripheral nerve sheath tumor: elongated spindle cells with kinking nuclei (hematoxylin and eosin × OI)



Figs 34.26A and B: (A) Rabdomyosarcoma: discrete round to oval cells with scanty cytoplasm (May-Grünwald-Giemsa × MP); (B) Rabdomyosarcoma: malignant round cells with scanty cytoplasm. The nuclei are round with opened up chromatin and prominent nucleoli (May-Grünwald-Giemsa × OI)

Embryonal Rhabdomyosarcoma

This is a primitive STS that simulates embryonic skeletal muscle. This is the most common type of RMS and mostly occurs in children below 10 years of age. The common sites of involvement are head and neck region, genitourinary system (urinary bladder, prostrate, paratesticular soft tissue) and extremities.

Cytology

Fine needle aspiration cytology smear of embryonal rhabdomyosarcoma shows predominantly two main cell types. One of these two types shows small round cells with scanty deep blue vacuolated cytoplasm. Nuclei are round, mildly pleomorphic having opened up fine chromatin and prominent nucleoli. These cells represent early rhabdomyoblasts. The second cell type is large with abundant cytoplasm. These cells display great variation in shape such as round, tadpole or ribbon-shaped. These cells

TABLE 34.2: Distinguishing features of different types of rhabdomyoblasts

Features	*Early rhabdomyoblast*	*Intermediate rhabdomyoblast*	*Late rhabdomyoblast*
Size	Small	Large	Same as intermediate
Shape	Round	Oval to polygonal	Oval to polygonal
Cytoplasm	Scanty deep blue	Abundant vacuolated	Abundant opaque
Nucleus	Large irregular single nuclei	Single to binucleation, prominent nucleoli	Pleomorphic nuclei

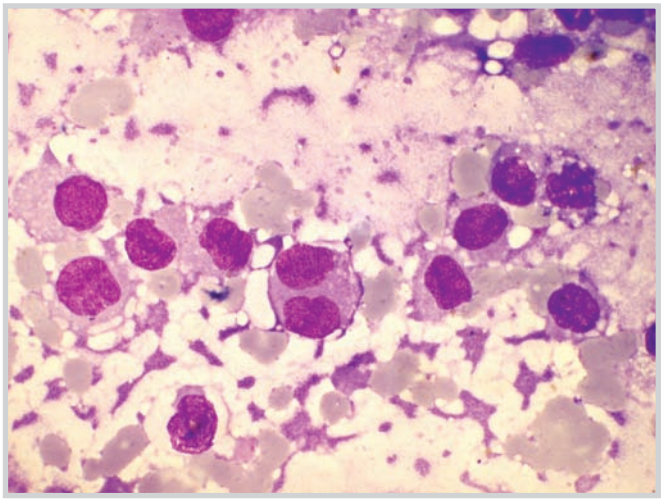


Fig. 34.27: Rabdomyosarcoma: discrete cells with bi-nucleation (May-Grünwald-Giemsa × OI)

represent intermediate to late rhabdomyoblasts. Frequent binucleation and multinucleation is noted. The nuclei of these cells are round with fine chromatin and prominent nucleoli.

Multinucleated giant cells and strap cells are also seen in embryonal RMS. Tigroid like background has also been described in RMS.

Pleomorphic Rhabdomyosarcoma

This is a relatively uncommon and highly malignant tumor. It predominantly occurs in the thigh region and is exclusively seen in adult patients.

Cytology

Fine needle aspiration cytology smear shows discrete and loosely cohesive large tumor cells. These cells show moderate amount of cytoplasm and severely pleomorphic large nuclei having multiple prominent nucleoli. Many multinucleated tumor giant cells are also seen. The smears also show increased mitotic activities.

Alveolar Rhabdomyosarcoma

Alveolar rhabdomyosarcoma is seen in all ages and does not show any predilection for children. It commonly occurs in extremities followed by paraspinal and perineal region.

Cytology

Cytology smear contains large number of early and small number of intermediate rhabdomyoblasts. Smear shows predominantly discrete small round cells with deep blue cytoplasm. Cytoplasm of the cells often shows small uniform vacuolations. Nuclei are round and mild to moderately pleomorphic with fine nuclear chromatin and prominent nucleoli. Cytoplasmic cross striations are usually absent. Alveolar RMS often shows large tumor giant cells with multiple nuclei. These nuclei are arranged in semicircular manner around the periphery of the cytoplasm.

Alveolar RMS shows a characteristic molecular cytogenetics t(2; 13) (q35; q14) in majority of the cases and a small subset shows translocation t(1; 13) (p36; q14).

Immunocytochemistry: The cells of RMS are positive for vimentin, desmin and myogenic differentiation (myo D)1.

Differential Diagnosis

Other small blue round cell tumor: Small blue round cell tumor, such as neuroblastoma, soft tissue Ewing's and lymphomas, come in the differential diagnosis of embryonal and alveolar RMS. The presence of strap cells is helpful in the identification of RMS.

Leiomyosarcomas[41]

Leiomyosarcomas usually occurs in adults. It is usually seen in retroperitoneum and mesentery. It may also occur in deep tissue of the extremities.

Cytology

Aspirate of LS shows cohesive clusters and small fascicles of cells. The discrete cells are also present (**Box 34.17**). The individual cells have moderate to abundant finely granular cytoplasm with elongated nuclei. The nuclei are cigar-shaped with blunt ends. Smears may also show many atypical cells with nuclear pleomorphism and multinucleated giant cells. The mitotic activity on cytology smear may not be reliable enough to distinguish LS from a leiomyoma. However, dissociated cell population, increased number of mitosis and nuclear pleomorphic are suggestive of LS (**Figs 34.28** to **34.30**).

Immunocytochemistry

The cells of LS are positive for SMA, muscle specific actin, desmin and caldesmon.

Differential Diagnosis

Other spindle cell tumor: Leiomyosarcoma should be distinguished from the other spindle cell tumors such as MPNST, fibrosarcoma, schwannoma, etc.

BOX 34.17 Leiomyosarcomas

- Bundles and small fascicles
- Dissociated cells
- Necrosis
- Spindle-shaped cells
- Abundant finely granular cytoplasm
- Cigar-shaped nuclei with blunt ends
- Nuclear atypia
- Multinucleated giant cells
- Increased mitotic activity

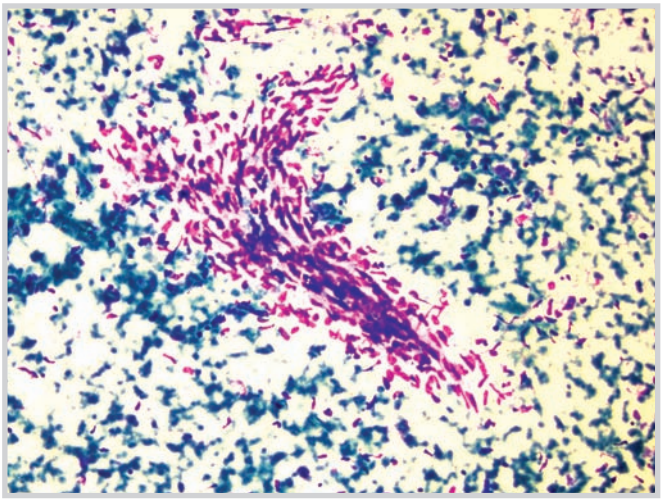


Fig. 34.28: Leiomyosarcoma: small fascicle of oval to spindle cells [May-Grünwald-Giemsa × low power (LP)]

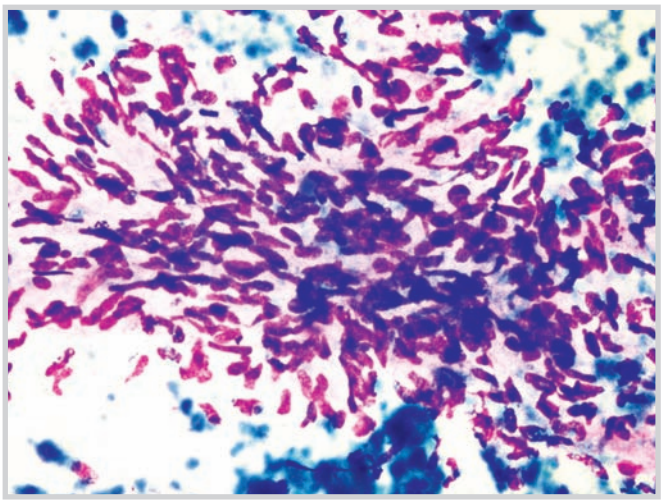


Fig. 34.29: Leiomyosarcoma: long rows of cells with elongated spindle cells having blunt ended cigar-shaped nuclei (May-Grünwald-Giemsa × MP)

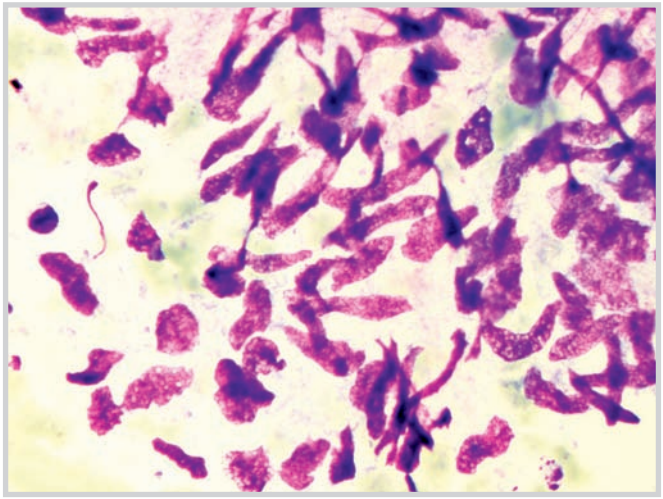


Fig. 34.30: Leiomyosarcoma: higher magnification showing cigar-shaped nuclei (May-Grünwald-Giemsa × OI)

BOX 34.18 **Hemangiopericytoma**

- Discrete and small fascicles
- Oval to elongated spindle cells
- Spindle-shaped nuclei with pointed ends
- Reticular nuclear chromatin
- Ropy collagenous material
- Naked nuclei

TUMOR OF VASCULAR ORIGIN

Hemangiopericytoma

It is a well circumscribed tumor of the pericyte. This is also known as solitary fibrous tumor. This tumor usually occurs in the fourth to fifth decade and preferentially distributes in the extremities, intra-abdominal region, mediastinum and orbit.

Cytology

Fine needle aspiration cytology smear are usually bloody with scanty to moderate cellularity (**Box 34.18**). The smear shows oval to elongated spindle cells that are arranged in short fascicles and also discretely. The tumor cells have scanty cytoplasm and elongated spindle-shaped nuclei with pointed ends. The nuclear chromatin is reticular. Mitotic count is variable. Background of the smear shows many naked nuclei and ropy collagenous material.

Immunocytochemistry

The tumor cells are positive for CD 34 and negative for desmin, CD 99 and S-100 immunostaining.

Glomus Tumor

These are rare tumors and represent less than 2% of all soft tissue tumors. The most common site is the distal extremities, particularly the subungual region of the fingers, the wrist and foot. However, it may occur in any part of the body. The tumor usually occurs in young and middle aged persons. Characteristically, the patient presents with a small red-blue painful nodule along with cold intolerance.

Cytology

Fine needle aspiration cytology is rarely performed in glomus tumor. The smear shows discrete round cells embedded in myxoid fibrillary background. The individual cells have abundant ill-defined cytoplasm with round bland nuclei. Nucleoli are inconspicuous (**Box 34.19**).

Immunocytochemistry

The tumor cells are positive for CD 31, CD 34 and SMA.

BOX 34.19 Glomus tumor

- Discrete round cells
- Myxoid fibrillary background
- Abundant ill-defined cytoplasm
- Round bland nuclei
- Nucleoli are inconspicuous

Hemangioma

This is a benign vascular neoplasm. However, evidences suggest that these are hamartomatous malformations rather than tumor. Hemangioma commonly occurs in children and majority of the tumors are seen in the head and neck region, and also in the extremities.

Cytology

Fine needle aspiration cytology of hemangioma is usually not indicated. In majority of the cases, clinical and radiological examinations are helpful in the diagnosis of hemangioma. FNAC yields blood from the lesion (**Box 34.20**). Smears are usually paucicellular. The cells are dissociated with oval to elongated spindle-shaped nuclei and pointed ends. The smears may also show variable number of histiocytes and fragments of capillaries.

Angiosarcoma

This is the malignant tumors of endothelial cells. It is a rare tumor and usually occurs in the elderly patient. Angiosarcoma commonly affects the deeper tissue of the lower extremities, arm, trunk and head and neck region.

Cytology

Fine needle aspiration cytology usually yields blood. Smears are paucicellular. There are scattered and occasional clusters of oval to elongated spindle cells (**Box 34.21**). The nuclei are spindle-shaped, moderately pleomorphic and hyperchromatic. Nuclear chromatin is fine. Smears also show signet ring-like cells with intracytoplasmic red blood cells.

Immunocytochemistry

The tumor cells are positive for CD34 and CD31.

TUMORS OF UNCERTAIN HISTOGENESIS

Synovial Sarcoma

The SS is a spindle cell tumor with distinct morphological entity. It represents about 5% of all STSs. This tumor has no relation with synovium. SS commonly occurs in the age group of 20–40 years.

BOX 34.20 Hemangioma

- Bloody smear
- Elongated spindle-shaped nuclei
- Capillary fragments
- Occasional histiocytes

BOX 34.21 Angiosarcoma

- Predominantly blood
- Scanty cellularity
- Spindle cells
- Moderate pleomorphism
- Hyperchromatic nuclei
- Signet ring cells with intracytoplasmic red blood cell (RBC)

The vast majority of the tumor is seen in the deep soft tissue of the extremity around the knee joints. However, SS may occur at any site. SS shows characteristic chromosomal alteration t(x;18) (p11;q11).

Cytology (Figs 34.31 to 34.35)

Biphasic: The biphasic SS shows admixture of two types of cells: (1) spindle cells and (2) epithelial like cells (**Box 34.22**). The epithelial cells are arranged as glands, tubules and papillae-like structures. These cells are round to oval shaped with moderate to abundant cytoplasm and round relatively monomorphic nuclei. The nuclear chromatin is fine with occasional prominent nucleoli.

The spindle cells are arranged in clusters with fascicles or whorled pattern. They are oval to elongated cells with scanty, delicate cytoplasm. The nuclei are spindle-shaped and monomorphic with irregular margin. Nuclei are hyperchromatic and often show single to multiple prominent nucleoli.

Monophasic Synovial Cell Sarcoma: The monophasic SS shows absence of any epithelial cells. The cells are predominantly arranged in small bundles and discretely. The individual cells have elongated spindle-shaped nuclei. Background of the smear may show fibrosis and calcified materials. FNAC smears of SS may show increased mitosis, mast cells and occasionally necrosis.[11,42]

Diagnostic Difficulties

Fibrosarcoma and other spindle cell sarcomas: Predominant population of spindle cells may pose diagnostic difficulty with fibrosarcoma, LS and other spindle cell sarcomas.

Clear Cell Sarcoma[43,44]

Clear cell sarcoma (CCS) is also known as malignant melanoma of the soft part. This is a STS with melanocytic differentiation.

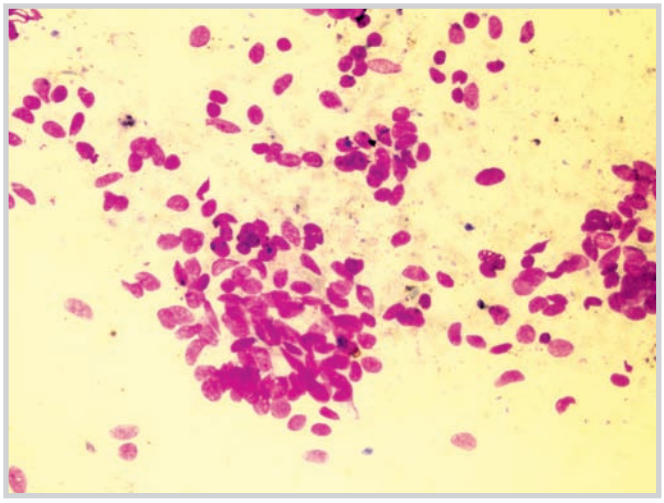


Fig. 34.31: Synovial sarcoma: clusters and discrete round and spindle cells with occasional tubular arrangement (May-Grünwald-Giemsa × MP)

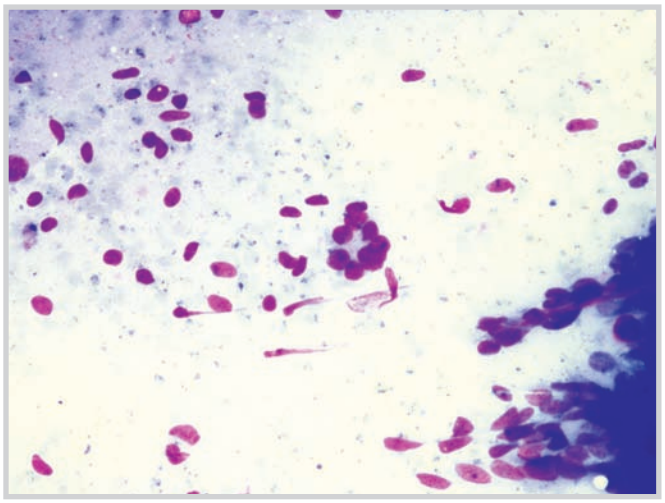


Fig. 34.34: Synovial sarcoma: tubular arrangement of the epithelial cells (May-Grünwald-Giemsa × HP)

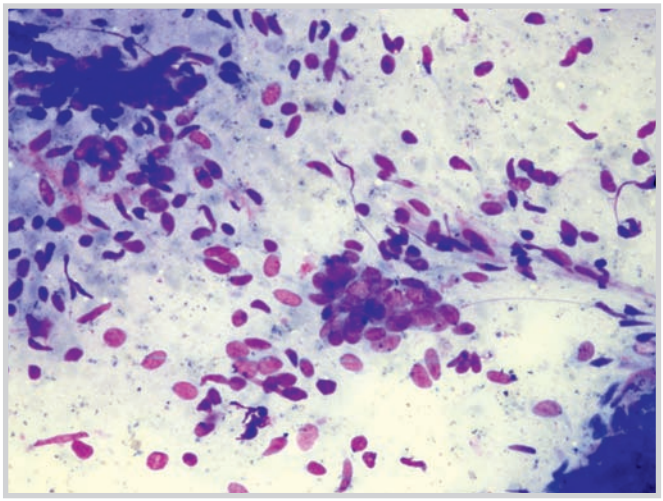


Fig. 34.32: Synovial sarcoma: round to oval epithelial cells and scattered spindle cells (May-Grünwald-Giemsa × HP)

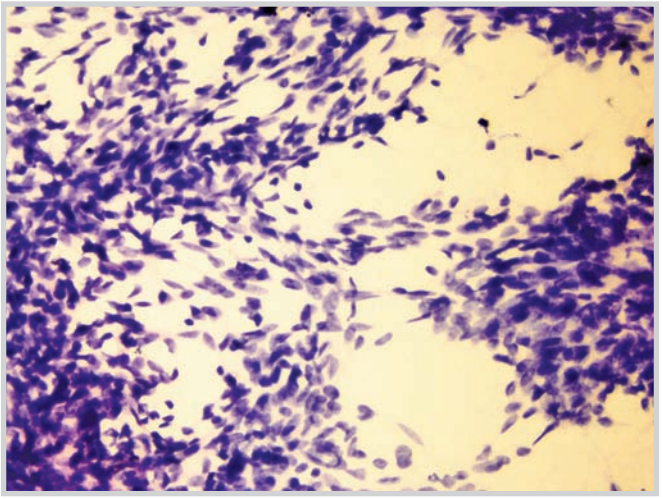


Fig. 34.35: Synovial sarcoma: predominant spindle cells in monophasic synovial sarcoma (hematoxylin and eosin × HP)

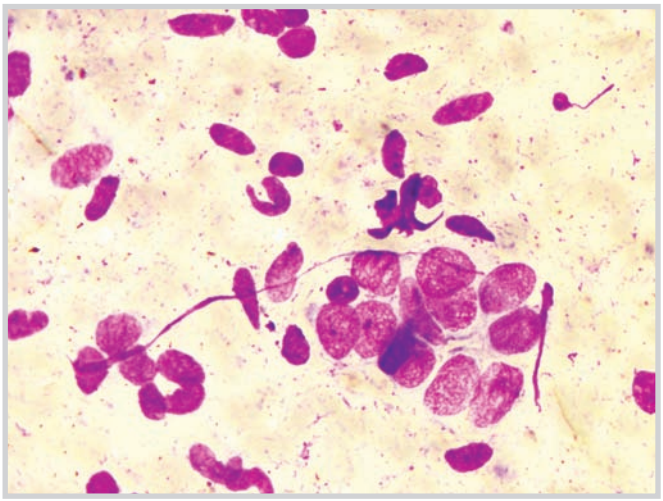


Fig. 34.33: Synovial sarcoma: higher magnification showing spindle cells and round cells (May-Grünwald-Giemsa × OI)

BOX 34.22 Synovial sarcoma

- Dual population of cells in biphasic synovial sarcoma (SS)
- Predominant spindle cell component in monophasic SS
- Spindle cells
  - Small fascicles and discretely arranged cells
  - Spindle-shaped nuclei
  - Hyperchromatic, irregular nuclear margin and prominent nucleoli
- Epithelial cells
  - Tubular/glandular arrangement
  - Round cells, monomorphic nuclei, prominent nucleoli
- Immunocytochemistry
  - Positive for cytokeratin (CK), Vimentin, epithelial membrane antigen (EMA), S-100 protein
- Molecular genetics
  - Characteristic chromosomal alteration t(x;18) (p11;q11)

BOX 34.23 Clear cell sarcoma

- Predominantly discrete and loosely cohesive cells
- Round to polygonal and spindle-shaped cells
- Abundant vacuolated cytoplasm
- Round, moderately pleomorphic and eccentrically placed nuclei
- Prominent nucleoli
- Intranuclear pseudoinclusions
- Multinucleated tumor giant cells
- Cytoplasmic pigmentation

The tumor typically involves the tendon and aponeuroses of the lower extremities followed by the hand and wrist. CCS is slow growing malignant tumor and commonly affects the young adults.

Cytology

The smear shows predominantly discrete and loosely cohesive cells. The tumor cells are round to polygonal and spindle-shaped with abundant vacuolated cytoplasm (**Box 34.23**). Occasional cells show cytoplasmic pigmentation. The nuclei of the cells are round, moderately pleomorphic and eccentrically placed with prominent nucleoli. Intranuclear pseudo inclusions are also noted. Multinucleated tumor giant cells and bi-nucleated cells are also present. CCSs are positive for human melanoma black (HMB-45) and S-100 protein.

Differential Diagnosis

- *Epithelioid sarcoma*: Epithelioid sarcomas (ES) are strongly positivity for keratin and negative for HMB-45 and S-100 protein.
- *Synovial sarcoma*: Monophasic SS may simulate CCS. However, the nuclei of the cells of SS show fine chromatin and indistinct nucleoli.
- *Metastatic carcinoma*: Metastatic carcinoma may also look like CCSs. Metastatic carcinomas are positive for CK and negative for HMB-45.

Epithelioid Sarcoma[45-47]

Epithelioid sarcoma is a distinct type of sarcoma of unknown etiology. ES usually occurs in the second to fourth decades of life and affects finger, hand, wrist and forearm.

Cytology

Fine needle aspiration cytology smears show both single and loose clusters of cells. The individual cells are round to polyhedral cells with dense eosinophilic cytoplasm (**Box 34.24**). Small intercellular spaces are also seen. The nuclei of the cells are large round and eccentric in position giving a plasmacytoid appearance. Nuclei show fine chromatin and prominent macronucleoli. Frequent binucleation is also seen. Smears may also show ill-formed granuloma like structures and inflammatory cells (**Figs 34.36** to **34.38**).

BOX 34.24 Epithelioid sarcoma

- Round to polyhedral cells
- Dense eosinophilic cytoplasm
- Large round and eccentrically located nuclei
- Prominent macronucleoli
- Epithelioid granuloma like structures
- Inflammatory cells

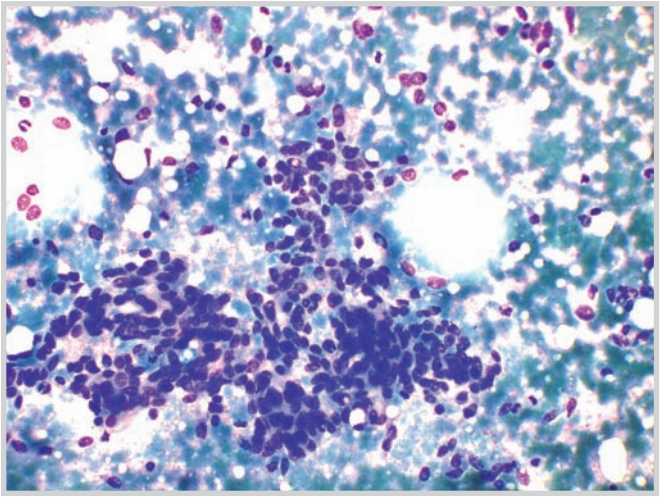


Fig. 34.36: Epithelioid sarcoma: loose clusters and discrete round to oval cells (May-Grünwald-Giemsa × MP)

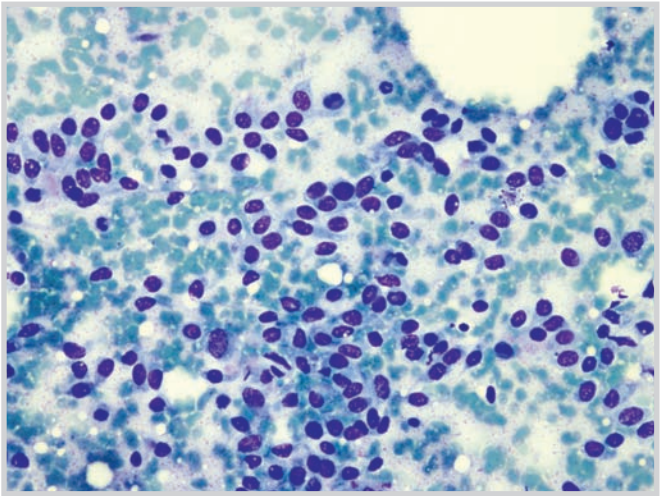


Fig. 34.37: Epithelioid sarcoma: discrete cells with mild to moderate amount of cytoplasm and central to eccentric nuclei (May-Grünwald-Giemsa × MP)

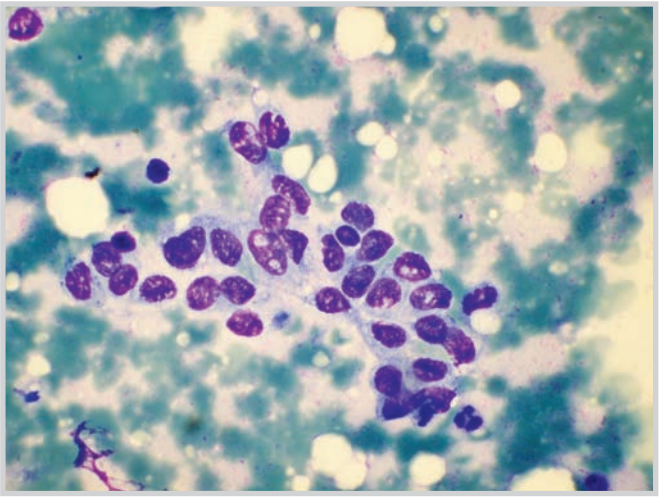


Fig. 34.38: Epithelioid sarcoma: higher magnification showing moderately pleomorphic cells with fine nuclear chromatin (May-Grünwald-Giemsa × HP)

Differential Diagnosis

- Clear cell saroma
- Poorly-differentiated metastatic carcinoma.

Alveolar Soft Part Sarcoma[48,49]

Alveolar soft part sarcoma is a rare tumor and represents only 0.5% of all STS. This tumor occurs mainly in adolescents and young adults between 15 years and 35 years of age. The tumor commonly affects the lower extremities preferentially deep tissue of thighs and buttock. Alveolar soft part sarcoma usually presents as painless slowly growing mass. However, early metastasis to lung is common.

BOX 34.25 Alveolar soft part sarcoma

- Single and loose clusters of cells
- Round to polygonal with well-defined cell border
- Abundant granular cytoplasm
- Nuclei are enlarged, round, central to eccentric in position
- Chromatin is stippled with large prominent nucleoli
- Occasional intranuclear inclusions

Cytology

The FNAC smear shows both clusters and discrete cells. The individual cells are round to polygonal with well-defined cell border (**Box 34.25**). The cytoplasm of the cell is abundant granular. Due to fragile cytoplasm, many bare nuclei are also noted in the background. Nuclei are enlarged, round, central to eccentric in position. Nuclear chromatin is stippled with large prominent nucleoli. Occasional intranuclear inclusions are also seen. Many cells show binucleation and multinucleation.

Immunostaining

The tumor cells are consistently positive to the antibody against carboxy terminal portion of transcriptor factor E3 (TFE3). The tumor cells are often positive for MyoD1 and desmin.

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CHAPTER 35

Skin

Chapter Contents

INTRODUCTION

The various skin lesions are easily accessible and a punch biopsy provides the diagnosis. Therefore, fine needle aspiration cytology (FNAC) of skin lesions is not much popular (**Box 35.1**). However like all other sites, FNAC can provide rapid and reliable diagnosis, particularly in differentiating inflammatory lesion from a tumor. It is also helpful in diagnosis of various metastatic lesions in skin. FNAC material from the neoplastic lesions in skin can be processed for various ancillary investigations such as cell block for immunocytochemistry in case of Markel cell carcinoma (MCC) or flow cytometry (FCM) in case of non-Hodgkin's lymphoma (NHL) of skin. A thorough knowledge of cytology of skin tumors may help to avoid false-positive diagnosis.[1-5]

Scrapping from the ulcer can be taken in small ulcerated lesions of skin. Larger lesions can be sampled by FNAC.

BOX 35.1 **Advantages of fine needle aspiration cytology (FNAC) of skin**

- Rapid
- Cost effective
- Reliably differentiates neoplastic lesion from inflammation
- Helps in identification of metastatic tumor in skin
- Ancillary investigation can be performed such as flow cytometry (FCM) and immunocytochemistry

NON-NEOPLASTIC LESIONS

Inflammation

Viral infection: Viral infection can best be diagnosed by simple scrapping from the base of the ulcer. The smears from molluscum contagiosum show classical "molluscum bodies" within the cytoplasm of the squamous cells replacing the nucleus to the periphery of the cell. The cytology smears from the herpes simplex infection show similar figure as described in Chapter 8 of this book. The smears show characteristic multinucleated cells with nuclear molding along with intranuclear large ground glass like inclusion.

Fungal infection: Scrapping from the ulcer base of the skin lesion may show fungal lesions. Individual morphology of the fungi studied by methylene blue stain is helpful to identify the type of fungus.

Bacterial infection: Bacterial infection gives rise to nonspecific inflammation. FNAC or scrapping of the lesion usually yields large number of inflammatory cells. In case of skin tuberculosis, FNAC smears may show epithelioid cell granulomas, multinucleated giant cells and lymphocytes. Ziehl-Neelsen (Z-N) stain should be done to show the presence of acid fast bacilli (AFB).

Parasitic infection: *Leishmania donovanii* in dermal Leishmaniasis is often identified in cytology smears. FNAC of the cysticercus infection yields clear fluid and the smears show tegument layer of the parasite (**Figs 35.1** and **35.2**). Background of the smear may show collection of histiocytes, lymphocytes and giant cell.

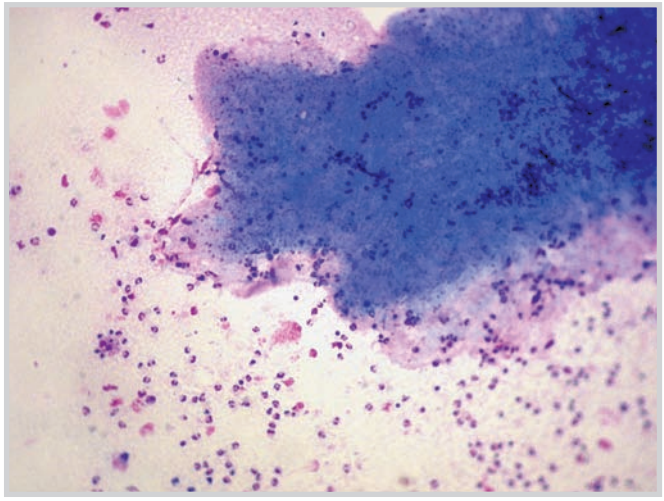

Fig. 35.1: Cysticercus infection: tegument layer of cysticercus along with many polymorphs and eosinophils in the background [May-Grünwald-Giemsa (MGG) stain × medium power (MP)]

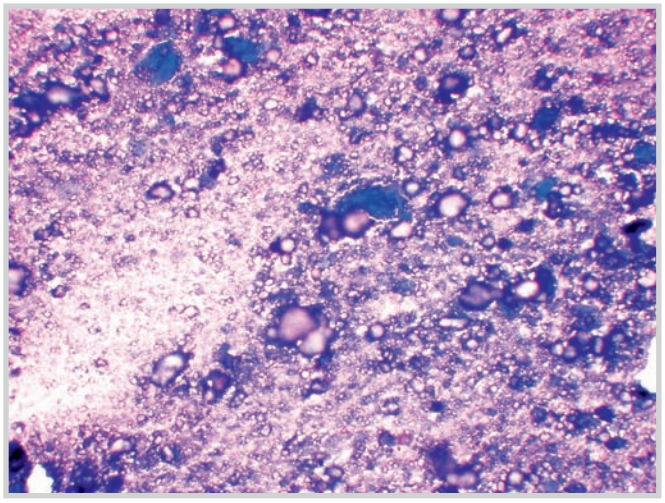

Fig. 35.3: Calcinosis cutis: granular calcified material (MGG × MP)

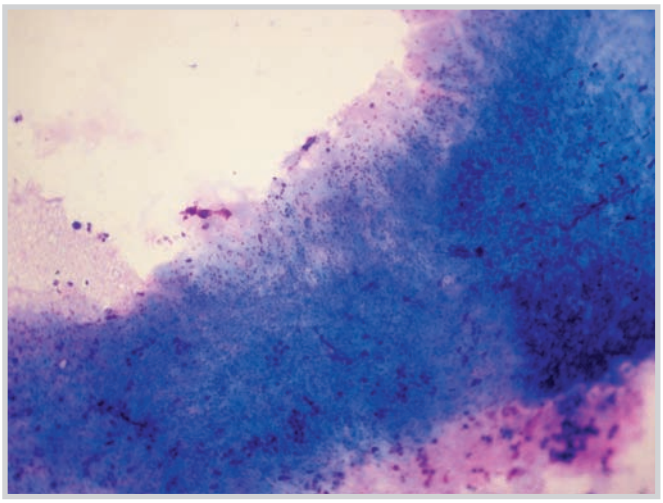

Fig. 35.2: Cysticercus infection: higher magnification shows detailed morphology of the tegument layer [MGG stain × oil immersion (OI)]

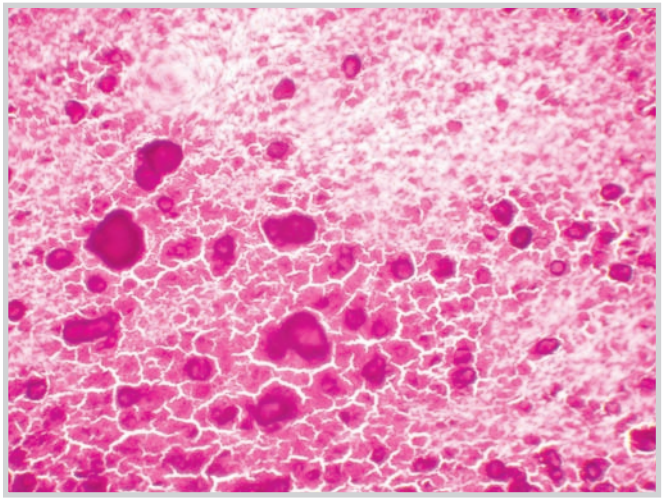

Fig. 35.4: Calcinosis cutis: acellular opaque calcified material [hematoxylin and eosin (H & E) stain × (MP)]

Rare Benign Diseases of Skin

Rarely calcinosis cutis, rheumatoid arthritis or sarcoidosis may present as a nodular skin lesion.[6]

Fine needle aspiration cytology of calcinosis cutis shows abundant granular calcified material along with occasional histiocytes (**Figs 35.3** and **35.4**).

Aspirates from the rheumatoid arthritis and sarcoidosis show granulomatous inflammation and smear contains multiple epithelioid cell granulomas, multinucleated giant cells and inflammatory cells. The granulomas of sarcoidosis are noncaseating.

Endometriosis (Fig. 35.5)

Endometriosis may present as nodular lesions in skin. Cytology smears show glandular epithelial cells, oval to spindle-shaped endometrial stroma and hemosiderin laden histiocytes. The characteristic clinical history of painful enlargement of the swelling during menstruation and bland nuclear morphology of the glandular epithelial cells are helpful in diagnosis of endometriosis.

Other Non-neoplastic Lesions

Epidermal Inclusion Cyst

Epidermal inclusion cyst is frequently aspirated (**Fig. 35.6**). FNAC of epidermal inclusion cyst yields thick whitish material. The smear shows abundant anucleated squamous cells. Occasionally, the cells may show reactive atypia and it may be difficult to distinguish well-differentiated squamous cell carcinoma from epidermal inclusion cyst. However, careful search may show

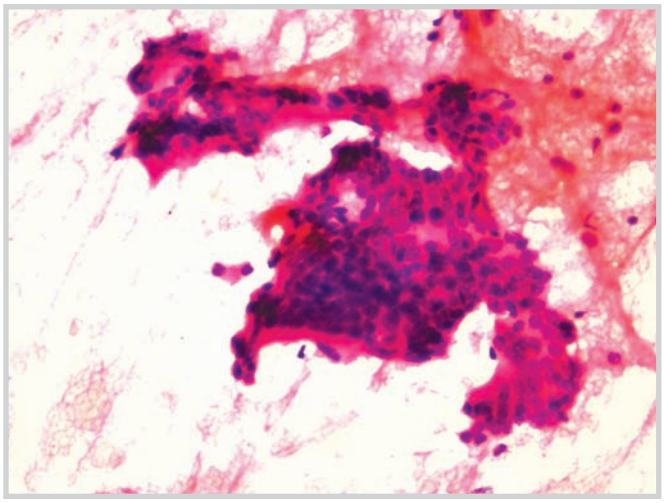

Fig. 35.5: Endometriosis: endometrial glandular epithelium [H & E × high power (HP)]

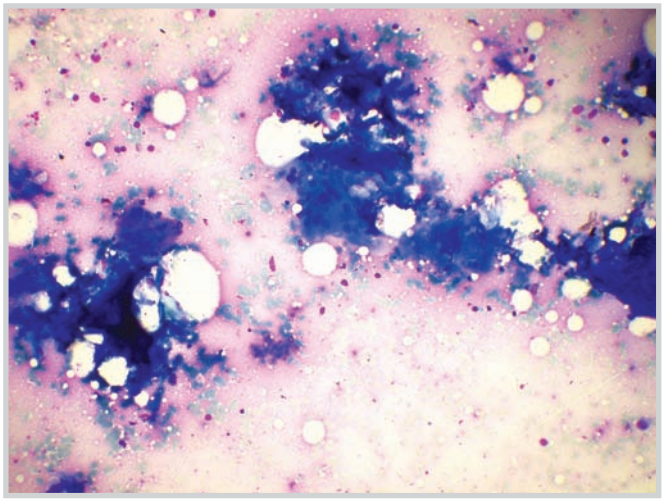

Fig. 35.7: Pilomatrixoma: ghost of squamous cells, multinucleated giant cells and calcified material (MGG × MP)

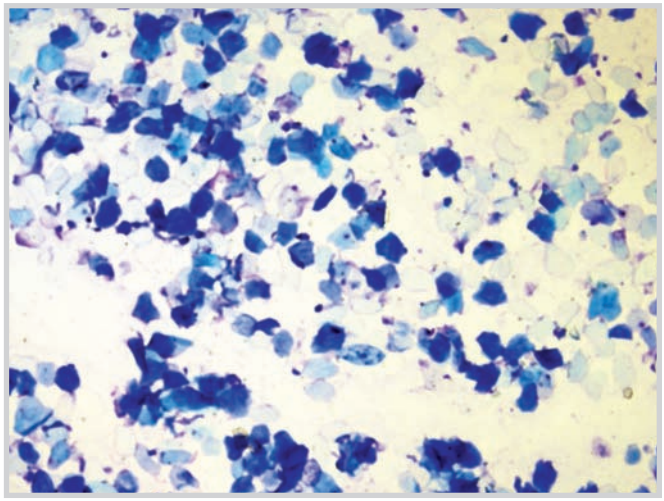

Fig. 35.6: Epidermal inclusion cyst: anucleated squamous cells (MGG × HP)

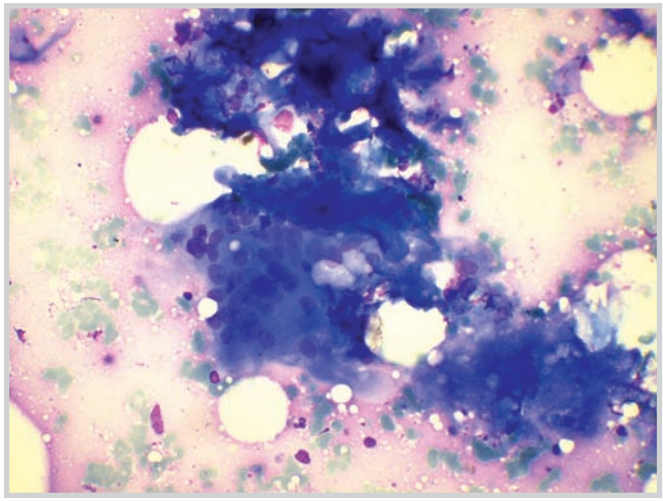

Fig. 35.8: Pilomatrixoma: higher magnification of the previous smears showing giant cells and calcified material (MGG × HP)

malignant cells in FNAC smear of squamous cell carcinoma. A good clinical history of primary malignancy may also be helpful. The smears also show many multinucleated giant cells.

Differential Diagnosis

Well-differentiated squamous cell carcinoma

NEOPLASTIC LESIONS OF SKIN

Benign Tumor of Skin Adnexa

Pilomatrixoma[4,7]

This is a benign adnexal tumor of the hair follicle and represents 0.2% of all routine dermatological samples. The tumor predominantly occurs in the young individuals and the common sites of involvement are head and neck region and upper extremities. The patient commonly presents as skin colored 1–3 cm diameter firm nodule.

Cytology (Figs 35.7 to 35.9)

The cytology smears show a mixed population of basaloid cells, ghost cells and multinucleated giant cells in a background of calcified material (**Box 35.2**). The basaloid cells have scanty bluish cytoplasm. The cell margin is usually ill-defined. The nuclei are round with high nucleus-to-cytoplasm (N/C) ratio and monomorphic. Nuclear chromatin is fine with single prominent nucleoli. The smear also shows many discrete anucleated squamous cells, known as ghost cells. They may often be present as small clumps. Many multinucleated giant cells are also seen. Background of the smear shows dark bluish, calcified material and myxoid material.

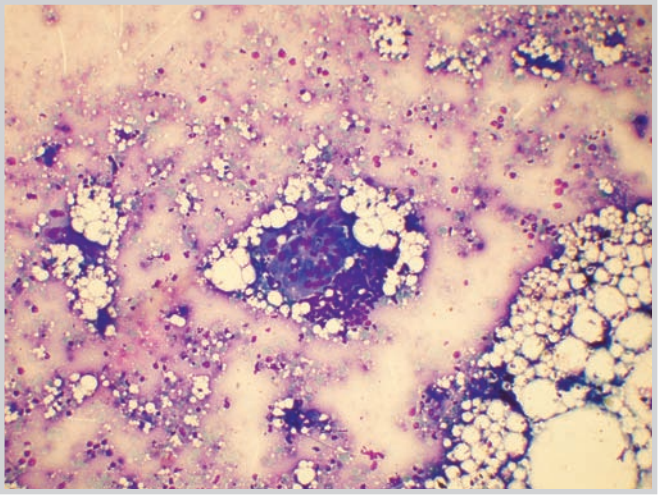

Fig. 35.9: Pilomatrixoma: cluster and discrete round monomorphic basal cells along with multinucleated giant cells (MGG × MP)

BOX 35.2 Pilomatrixoma

- Anucleated squamous cells: ghost cells
- Monomorphic round basaloid cells
  - Round
  - Scanty ill-defined cytoplasm
  - Monomorphic nuclei
  - Fine chromatin
- Multinucleated giant cells
- Calcified material

Differential Diagnosis
- Metastatic squamous cell carcinoma
- Epidermal inclusion cyst
- Foreign body reaction

Chondroid Syringoma

This is a benign skin adnexal tumor analogous to mixed tumor of the salivary gland. It commonly occurs in adults. Head and neck regions are commonly affected by chondroid syringoma.

Cytology

Fine needle aspiration cytology of chondroid syringoma shows discrete and small clusters of round to oval epithelial cells embedded in purple fibrillary chondromyxoid material (**Box 35.3**). The cells have moderate amount of dense cytoplasm with central to eccentric round nuclei. Nuclear chromatin is fine with single prominent nucleoli.[8]

Differential Diagnosis

Basal cell carcinoma (BCC)

BOX 35.3 Chondroid Syringoma

- Discrete and small clusters of round to oval epithelial cells
- Round cells
- Well-defined cytoplasmic margin
- Round nuclei with fine chromatin
- Prominent nucleoli
- Fibrillary chondromyxoid material

BOX 35.4 Cylindroma

- Homogenous purple colored globules
- Basaloid cells
  - Round cells
  - Scanty cytoplasm
  - Monomorphic round nuclei
  - Hyperchromatic nuclei

Cylindroma

Cylindroma usually occurs in the scalp. Aspiration cytology smears simulate adenoid cystic carcinoma of the salivary gland. FNAC smear shows multiple homogenous purple colored hyaline globules [May-Grunwald-Giemsa (MGG) stain] surrounded by round to oval epithelial cells (**Box 35.4**). These cells have scanty cytoplasm and round monomorphic hyperchromatic nuclei.

Eccrine Spiradenoma

This is a benign skin adnexal tumor. FNAC smear of eccrine spiradenoma simulates adenoid cystic carcinoma. The cytology smear shows stromal matrix material surrounded by epithelial cells giving a rosette-like arrangement (**Box 35.5**). The epithelial cells are also arranged as cell balls. The epithelial cells are cuboidal cells with scanty cytoplasm having round nuclei and inconspicuous nucleoli. In addition, the smears also show discrete myoepithelial cells and scattered lymphocytes.

Hidradenoma

This is a benign skin adnexal tumor of eccrine sweat gland. This is also known as clear cell hidradenoma. This tumor commonly occurs in adult. Hidradenomas commonly involves the scalp, trunk and proximal extremities.

Cytology[9, 10]

Fine needle aspiration cytology smear shows cohesive clusters and tubule-like structures of cells. Occasional scattered cells with naked nuclei are also seen (**Box 35.6**). The cells are cuboidal to polyhedral in shape with scanty cytoplasm. The nuclei are monomorphic round with bland chromatin and inconspicuous nucleoli. Background of the smear shows scattered dense round metachromatic-staining globules.

BOX 35.5 **Eccrine spiradenoma**

- Stromal matrix material surrounded by epithelial cells
- Cuboidal epithelial cells
  - Scanty cytoplasm
  - Round nuclei and inconspicuous nucleoli
- Myoepithelial cells
- Lymphocytes

BOX 35.6 **Hidradenoma**

- Cohesive clusters and tubules
- Cuboidal to polyhedral in cells
- Scanty cytoplasm
- Monomorphic round nuclei
- Bland chromatin
- Dense round metachromatic-staining globules

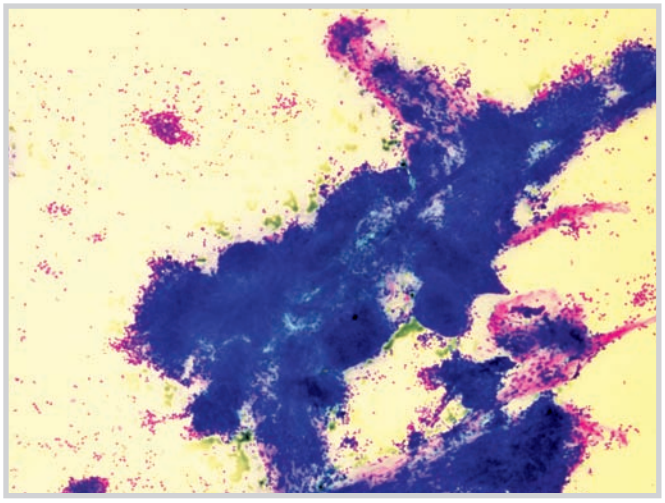

Fig. 35.10: Syringocystadenoma papilliferum: pultiple papillary structures [MGG × low power (LP)]

Syringocystadenoma Papilliferum

Syringocystadenoma papilliferum is a rare cutaneous adnexal neoplasm (**Figs 35.10** to **35.14**). The average age at the time of presentation is 28 years. The tumor commonly occurs in the trunk and scalp region. FNAC smears show multiple papillae-like structures along with discrete monomorphic epithelial cells and occasional spindle cells. Smears may also show chronic inflammatory cells.

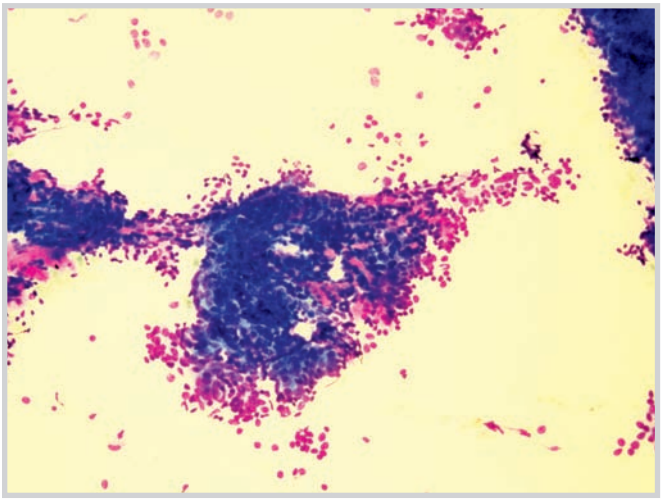

Fig. 35.11: Syringocystadenoma papilliferum: papillary cluster along with round to oval epithelial cells and elongated spindle cells (MGG × MP)

MALIGNANT TUMORS OF SKIN

Basal Cell Carcinoma[11-13]

Basal cell carcinoma is also known as trichoblastic carcinoma. This is the most frequent primary skin cancer. It predominantly affects the sun-exposed skin area. BCC is commonly seen in adults and usually affects in the head and neck region as slowly growing ulcerated nodule or papule.

Cytology (Figs 35.15 and 35.16)

Fine needle aspiration cytology smears show small tight cluster of cells (**Box 35.7**). The cells are often arranged as palisading manner around the periphery of the clusters. The tumor cells contain scanty cytoplasm with very high N/C ratio. The nuclei of the cells are round, monomorphic and hyperchromatic. Occasional cells may show small distinct nucleoli.

Differential Diagnosis

- Small cell carcinoma
- Markel cell carcinoma
- Poorly-differentiated skin adnexal carcinoma

Sebaceous Carcinoma[14-16]

Sebaceous carcinoma (SC) is commonly seen in the lacrimal gland of eye. It usually occurs in elderly adults and commonly presents as painless mass in the eyelid. It may be mistaken clinically as chalazion blepharitis, or conjunctivitis.

Cytology

Fine needle aspiration cytology smears show predominantly discrete cells and tight three-dimensional clusters. Occasional glandular arrangements are also seen. Background of the smear shows fatty material and keratin debris (**Box 35.8**). The individual cells are large with moderate to abundant amount of finely vacuolated cytoplasm and well-defined cytoplasmic margin. Cytoplasmic vacuoles are positive for Oil Red O. Nuclei are central in position and round to oval with moderate pleomorphism. Occasional nuclei may be notched or indented. Smears may show individual cell necrosis and background necrosis. Occasional multinucleated giant cells may also be seen.

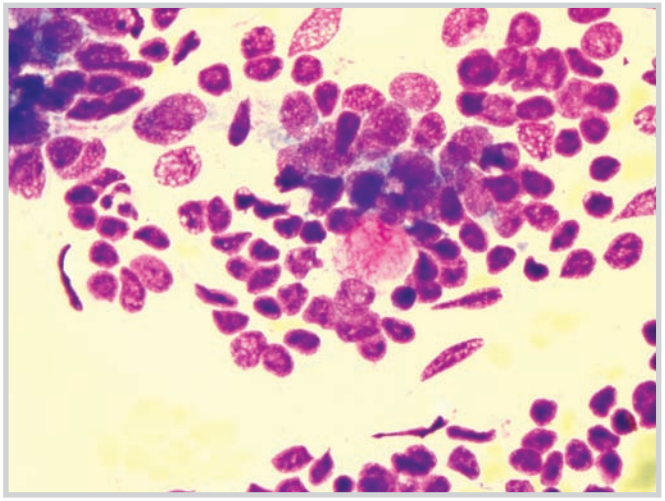

Fig. 35.12: Syringocystadenoma papilliferum: higher magnification showing better morphology of the cells. The cells are round with scanty cytoplasm having monomorphic bland nuclei. Cells occasionally arranged around pinkish globules. (MGG × OI)

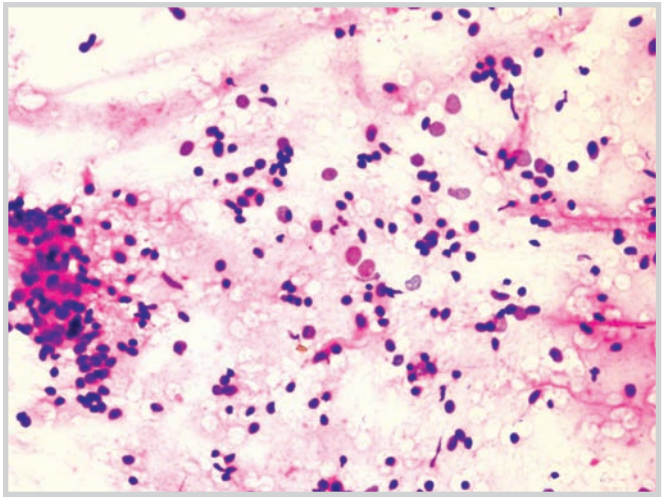

Fig. 35.14: Syringocystadenoma papilliferum: nuclear morphology is better seen in hematoxylin and eosin stained smear (H & E × HP)

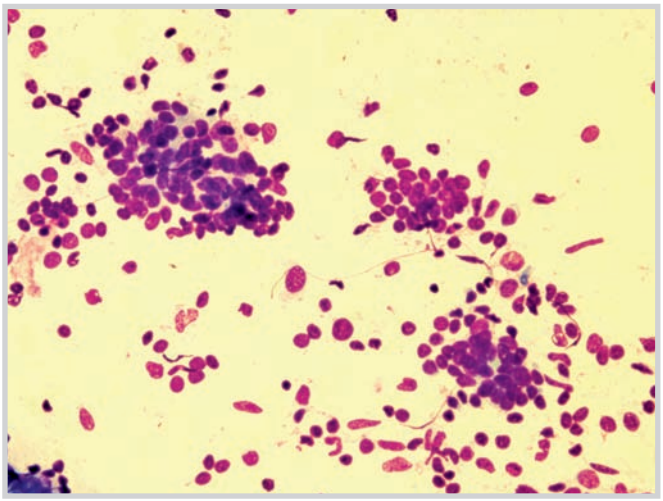

Fig. 35.13: Syringocystadenoma papilliferum: syringocystadenoma papilliferum: discrete round and spindle-shaped cells along with few inflammatory cells (MGG × HP)

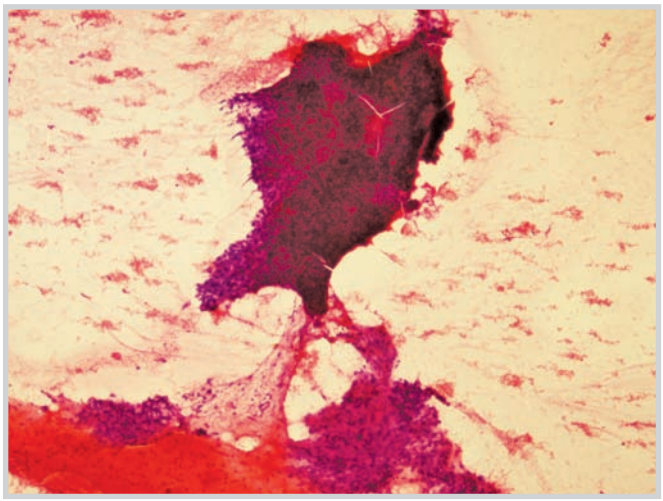

Fig. 35.15: Basal cell carcinoma: multiple cohesive clusters of round cells (H & E × MP)

Differential Diagnosis

- Pagetoid malignant melanoma
- Paget's disease

Malignant Melanoma[4,17,18]

Malignant melanomas develop from the melanocytes of the skin and are the aggressive cancers. They are commonly noted in the sun-exposed area such as head and neck region and lower extremities. The tumor usually occurs in adults but may also develop before puberty. Malignant melanoma usually develops spontaneously or occasionally from the pigmented nevi.

Cytology (Figs 35.17 and 35.18)

Fine needle aspiration cytology of primary melanoma is not common. Most of the cases are aspirated from the metastatic sites. The smears show predominantly dyscohesive cells (**Box 35.9**). The individual cells have abundant cytoplasm with central to eccentric large round moderately pleomorphic nuclei. The nuclei often show single to multiple prominent nucleoli. Macronucleoli are often seen. Intranuclear cytoplasmic inclusions are also noted. Dark-brown melanin pigment in the cell is characteristic for the diagnosis of melanoma. Occasionally, malignant melanoma shows predominant spindle cell morphology. The spindle cells are arranged in interlacing fascicles and whorls and also discretely. The individual cells contain variable amount of cytoplasm with

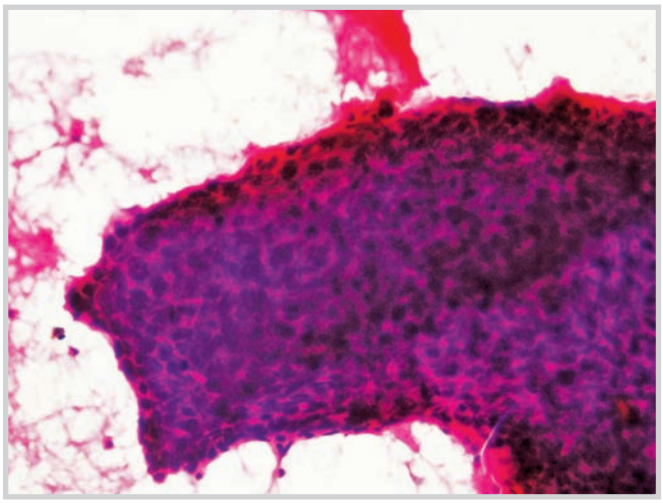

Fig. 35.16: Basal cell carcinoma: round cells with hyperchromatic nuclei arranged as palisading manner (H & E × HP)

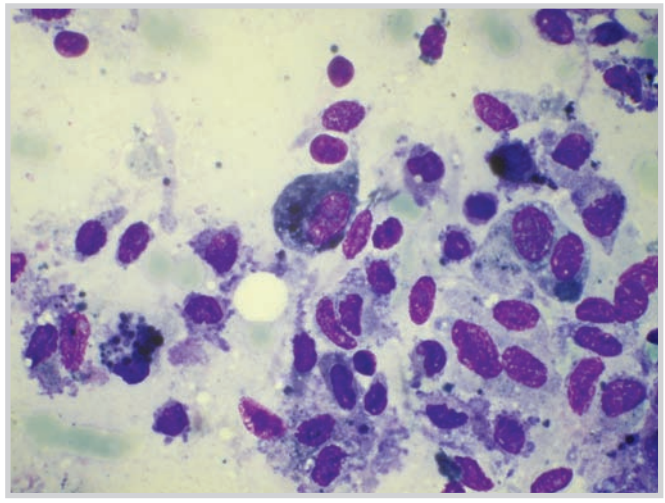

Fig. 35.17: Malignant melanoma: discrete moderately pleomorphic cells with melanin in the cytoplasm (MGG × MP)

BOX 35.7 Basal cell carcinoma

- Tight clusters of round cells
- Peripheral palisade like arrangement
- Round cells
- Scanty cytoplasm
- Very high N/C ratio
- Hyperchromatic round nuclei
- Occasional prominent nucleoli

BOX 35.8 Sebaceous carcinoma

- Discrete cells and tight three-dimensional clusters
- Large cells with well-defined borders
- Abundant amount of finely vacuolated cytoplasm
- Nuclei are central
- Round to oval with moderate pleomorphism
- Individual cell necrosis and background necrosis
- Multinucleated giant cells

bipolar elongated cytoplasmic process. The cytoplasm of these cells often shows dark-brown melanin pigment. Nuclei are moderately pleomorphic oval to elongated having finely granular chromatin. Binucleation and multinucleation are also noted. In the absence of melanin, malignant melanoma with spindle cells is difficult to diagnose only on cytology smear.

Immunocytochemistry

Melanoma cells are positive for HMB-45, melanoma antigen (melan-A) and S-100 protein. HMB-45 is more specific than S-100 protein immunostaining. Melan-A, a melanocytic differentiation antigen is present in 80% of malignant melanoma cases.

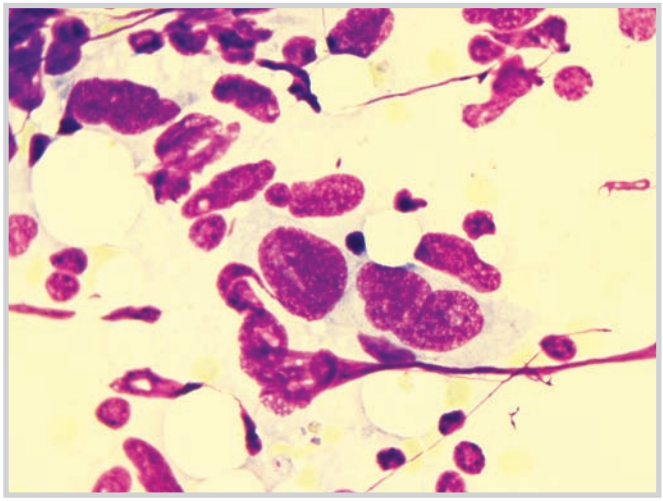

Fig. 35.18: Malignant melanoma: cells with markedly pleomorphic nuclei having prominent nucleoli (MGG × HP)

BOX 35.9 Malignant melanoma

- Discrete round cells
- Moderate cytoplasm with dark-brown pigment
- Moderately pleomorphic nuclei
- Many binucleated and multinucleated cells
- Macronucleoli
- Intranuclear inclusions
- Spindle cells
- Human Melanoma Black (HMB)-45 and S-100 positive

Diagnostic Difficulties

- Carcinoma
- Non-Hodgkin's lymphoma
- Sarcoma

Squamous Cell Carcinoma

It is the malignant tumor of the keratinocytes of the epidermis with squamous differentiation. The tumor usually involves the exposed area of the body such as forehead, face and hand. The patient usually presents as shallow ulcer with indurated margin.

Cytology

Smear shows discrete tumor cells. The tumor cells are polyhedral with moderate amount of cytoplasm. Cytoplasmic orangeophilia are seen in Papanicolaou's staining (**Box 35.10**). Nuclei are enlarged with high N/C ratio and moderately pleomorphic. Nuclear chromatin is condensed with inconspicuous nucleoli. Occasional fiber cells and tadpole-like cells may also be seen. In addition, mature looking anucleated cells may also be seen.

Malignant Sweat Gland Tumor[19,20]

Cytology smear of malignant sweat gland tumor shows abundant cells. The cells are discrete and also with tubular arrangement. The tumor cells show abundant pale cytoplasm with central mildly pleomorphic nuclei. Cytological diagnosis of malignant sweat gland tumor is often difficult. Detailed clinical history may be helpful in diagnosis of malignant sweat gland tumor.

Markel Cell Carcinoma[21,22]

Markel cell tumor is a relatively rare primary malignant tumor of the skin. Most of the tumor arises on the sun-exposed skin area and head and neck regions are commonly affected.

Cytology (Figs 35.19 and 35.20)

Fine needle aspiration cytology smear of Markel cell carcinoma shows predominant discrete small round cells and occasional pseudorosette-like arrangement (**Box 35.11**). The tumor cells are small round with scanty deep blue cytoplasm. The nuclei are small round with fine chromatin and multiple small nucleoli. Occasional cell shows nuclear molding. Mitosis is usually frequent. Many histiocytes may also be noted in the background.

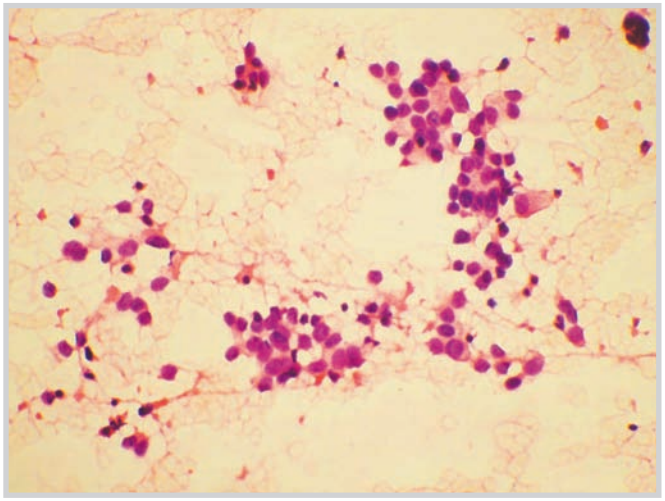

Fig. 35.19: Markel cell carcinoma of skin: discrete and occasional rosette-like arrangement of small round cells with scanty cytoplasm having round monomorphic nuclei (H & E × MP)

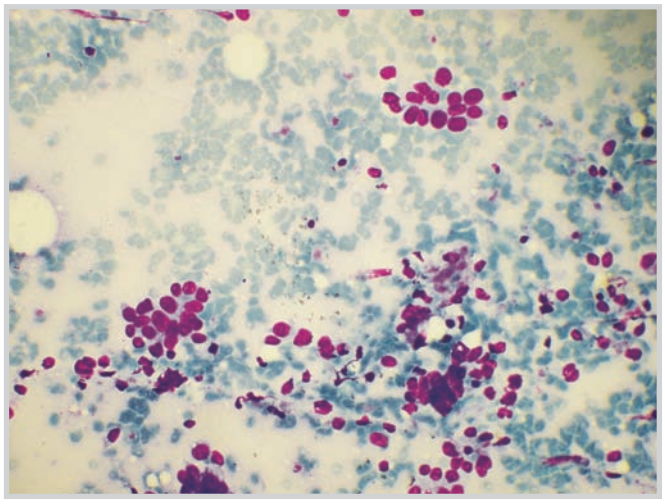

Fig. 35.20: Markel cell carcinoma of skin: discrete and loose clusters of small round cells (MGG × MP)

BOX 35.10 **Squamous cell carcinoma**

- Polyhedral cells
- Abundant cytoplasm
- Orangeophilia in Papanicolaou's stain
- High N/C ratio
- Moderate nuclear pleomorphism
- Anucleated squamous cells
- Fiber and tadpole cells

BOX 35.11 **Markel cell tumor**

- Discrete cells
- Occasional pseudorosette
- Scanty deep blue cytoplasm
- Round nuclei, fine chromatin and occasional molding

Immunocytochemistry

Markel cell carcinomas are positive for cytokeratin-20(CK20), neuron-specific enolase (NSE), epithelial membrane antigen (EMA) and S-100. The cells are also positive for CD117. In contrast, small cell carcinomas are negative for CK20 and positive for TTF-1.

Differential Diagnosis

- Small cell carcinoma
- non-Hodgkin's lymphoma

METASTASIS[23,24]

Skin often shows metastasis from various sources. The common sources of metastasis are from lung, breast, large intestine, melanoma and kidney. Metastatic soft tissue tumor is also not rare (**Fig. 35.21**). Occasionally, solitary metastatic skin lesion may simulate a primary skin tumor on clinical examination. Cytomorphology of metastatic carcinoma of skin depends on the type of primary malignancy. Some of the metastatic tumors are readily identifiable from the cytomorphology such as signet ring carcinoma of the stomach and clear cell carcinoma of kidney.

Differential Diagnosis

- Endometriosis
- Primary skin tumor.

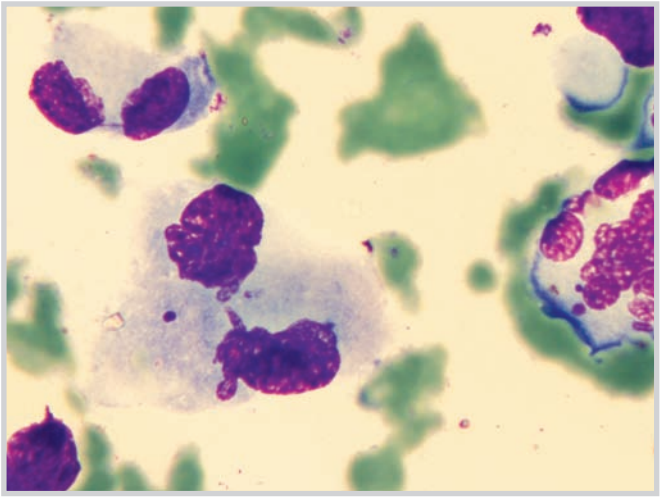

Fig. 35.21: Metastatic leiomyosarcoma: large pleomorphic multinucleated cells from a uterine leiomyosarcoma to skin (MGG ×HP)

Hematopoietic Malignancy

Certain lymphomas often involve the skin such as anaplastic large cell lymphomas in pediatric population and mycosis fungoides in adult population. The cytomorphology of these lymphomas is same as that of lymph node. Leukemia also involves the subcutaneous tissue of skin. Cytology smear shows myeloid series of cells and blasts.

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CHAPTER 36

Bone

Chapter Contents

INTRODUCTION

Fine needle aspiration cytology (FNAC) for the primary diagnosis of bone tumors is still not well-popularized and there are many concerns regarding its use.[1-5] The cytologists are not aware of the detailed morphology of many primary benign lesions of bone and at times it may be difficult to provide the exact histological type of the bone tumor. Ancillary investigations, particularly immunocytochemistry and molecular genetics, are not always possible from FNAC material. Moreover, FNAC may not yield adequate diagnostic material particularly in benign bony lesions. However, in case of malignant bone lesion, the needle can easily pierce the bone and adequate material can be aspirated. The insufficiency rate of FNAC of bone lesions varies from 8% to 31%.[6-8] The adequacy of FNAC material depends on the type of the lesion and radiological appearance. Overall low yield of material is seen predominantly in cystic lesion with thick intact bone cortex (**Box 36.1**). Such as aneurysmal bone cyst (ABC) and simple bone cyst often yields poor diagnostic material. Densely sclerotic bony lesions such as osteoid osteoma and osteoblastoma may also yield insufficient material for diagnosis. Similarly, bony lesions which are predominantly calcified, cartilaginous or necrotic, may also yield nonrepresentative material. Careful selection of the patient for FNAC helps to reduce the insufficiency rate. The diagnostic accuracy of FNAC of bone varies from 80% to 93%.[1-5,9,10] The accuracy rate depends on the experience of the cytologist and careful correlation of radiological pictures and FNAC findings.

BOX 36.1 **Poor diagnostic yield of bone fine needle aspiration cytology (FNAC)**

- Predominant cystic lesion
- Thick intact cortex
- Densely sclerotic lesion
- Predominant cartilaginous lesion or necrotic lesion

To increase the diagnostic accuracy of FNAC of bone, the cytologists require (1) the detailed clinical history, (2) anatomic locations of the tumor and (3) the radiological findings.

NORMAL CELLS

- *Osteoblasts* ***(Fig. 36.1)***: Osteoblasts are round cells with moderate to abundant reddish granular cytoplasm. The nuclei are eccentric in position and give an appearance of coming out from the cell. The nuclear chromatin is fine with small nucleoli.
- *Osteoclast* ***(Fig. 36.2)***: Osteoclasts are large cells giant cells with abundant cytoplasm that contain about 30–50 nuclei.
- *Chondrocytes* ***(Fig. 36.3)***: Chondrocytes are always present in cluster and are embedded in the bright red to magenta colored chondroid material. The cells have small round pale nuclei with clear halo around the nuclei.
- World Health Organization (WHO) classified bone tumors as mentioned in **Box 36.2**.[11]

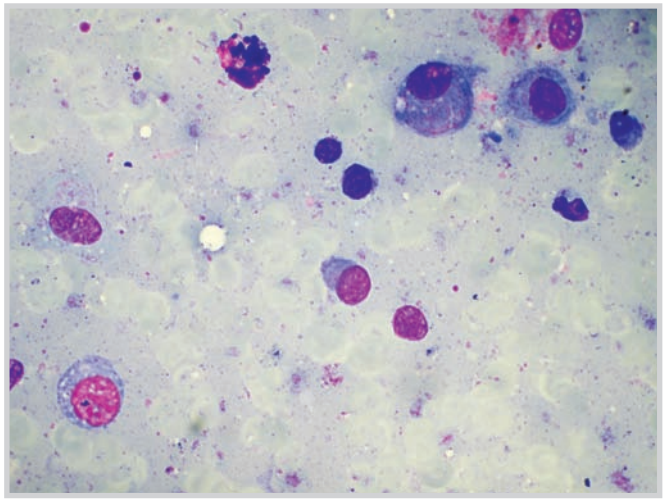

Fig. 36.1: Osteoblasts: round cell with abundant cytoplasm and peripherally placed nuclei [May-Grünwald-Giemsa (MGG) × high power (HP)]

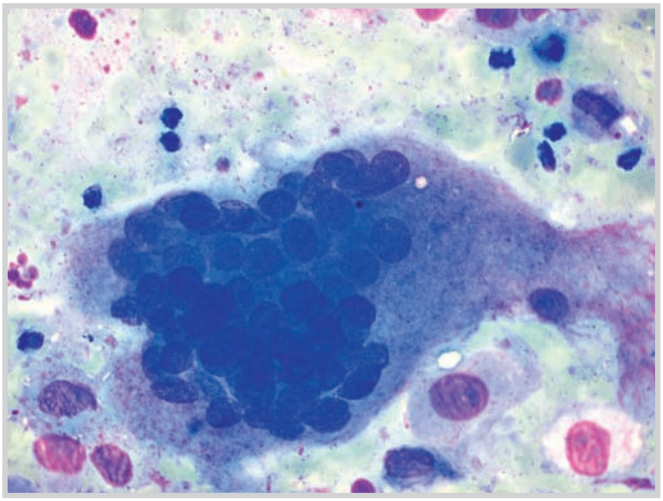

Fig. 36.2: Osteoclasts: large cell with at least 30 nuclei (MGG × HP)

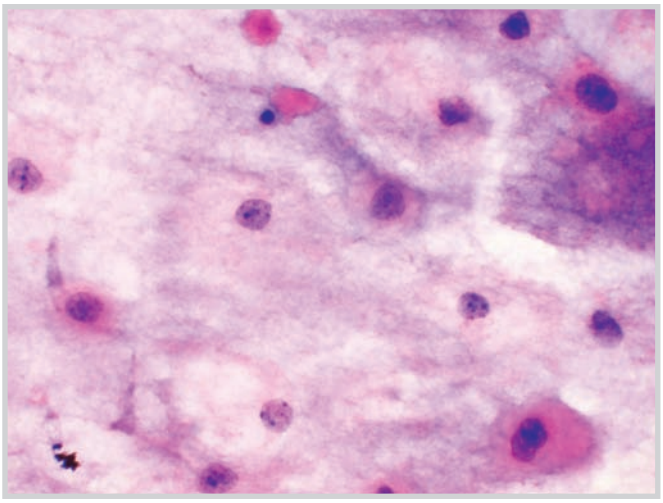

Fig. 36.3: Chondrocytes: chondroid cells in the background of chondroid material [hematoxylin and eosin (H & E) × HP]

BOX 36.2 **World Health Organization classification of bone tumors**

Neoplastic

Osteogenic tumor

Benign:
- Osteoid osteoma
- Osteoblastoma

Malignant:
- Osteosarcoma:
  - Conventional
  - Chondroblastic
  - Fibroblastic
  - Osteoblastic
  - Telangiectatic
  - Small cell
  - Low-grade central
  - Secondary
  - Parosteal
  - Periosteal

Cartilage forming

Benign:
- Chondroma
- Chondromyxoid fibroma
- Chondroblastoma

Malignant:
- Chondrosarcoma
  - Central, primary and secondary
  - Peripheral
  - Dedifferentiated
  - Mesenchymal
  - Clear cell

Giant cell tumor

Notochodal tumor: Chordoma

Vascular tumor: Hemangioma, angiosarcoma

Smooth muscle tumor: Leiomyoma, leiomyosarcoma

Fibrogenic tumor: Desmoblastic fibroma, fibrosarcoma

Marrow tumor:
- Plasma cell tumor
- Lymphoma

Ewing's tumor or peripheral neuroectodermal tumor

Miscellaneous: Aneurysmal bone cyst, Langerhans's cell histocytosis

BONE FORMING TUMOR

Osteoblastoma

This is a rare bone forming benign tumor that represents only 1% of bone tumors. The tumor commonly affects young male and the age range varies from 10 years to 30 years. The common sites of osteoblastoma are posterior part of the spine, sacrum, femur and proximal part of tibia. Radiography of the tumor shows lytic well-circumscribed oval or round defect confined by a periosteal shell.

Cytology

Fine needle aspiration cytology smears of osteoblastoma show clusters and discrete osteoblasts and osteoclastic giant cells

BOX 36.3 Osteoblastoma

- Osteoid material
- Osteoblasts
- Osteoclasts
- Spindle cells in small fascicles

BOX 36.4 Osteosarcoma

- Discrete and clusters of round cells
- Cells with abundant reddish granular cytoplasm resembling osteoblast
- Moderate to marked nuclear enlargement and pleomorphism with coarse chromatin
- Multinucleated tumor giant cells
- Spindle cells
- Pinkish osteoid material

(**Box 36.3**). Osteoblasts show abundant reddish granular cytoplasm and single to double nuclei. The background of the smear shows pinkish osteoid material and clusters of monomorphic spindle cells. The spindle cells are often arranged in small fascicles and embedded in pinkish stromal material.[12,13]

Osteosarcoma[14-16]

Osteosarcoma is the primary intramedullary high-grade malignant tumor of the bone. It commonly occurs in the second and third decade of life and 60% of the tumor occurs before the age of 25 years. Osteosarcoma commonly arises from the metaphyseal area of the long bone such as lower end of femur and upper end of tibia and fibula. A small percentage of osteosarcoma occurs in diaphyseal area of bone. The common sites of non-long bone involvement of osteosarcoma are jaw bones, pelvic bones and skull.

The patients present with nonspecific pain along with bony swelling and physical examination reveals a painful tender mass. Radiological examination shows osteolytic, osteoblastic or mixed lesion that breaks the cortical bone and infiltrates into the surrounding soft tissue. The salient cytological features of osteosarcoma are mentioned in **Box 36.4**.

Cytology (Figs 36.4 to 36.7)

Cytology smear of conventional osteosarcoma shows abundant cells. The cells are predominantly oval to polygonal with eccentric nuclei. The amount of cytoplasm varies from scanty to moderate in amount. The cytoplasm often shows reddish granularity and small vacuolations in May-Grünwald-Giemsa (MGG) stain. The nuclei are markedly pleomorphic with coarse chromatin and inconspicuous to prominent nucleoli. Many binucleated and multinucleated tumor cells are seen. Background of the smear shows numerous mitotic activities and necrotic tissue. The smears of osteosarcoma cases show pinkish granular osteoid material.

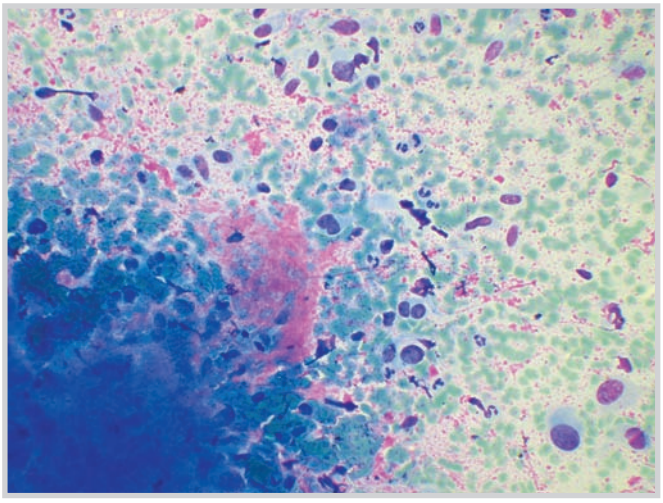

Fig. 36.4: Osteosarcoma: discrete round and spindle-shaped malignant cells along with pinkish osteoid material (MGG × HP)

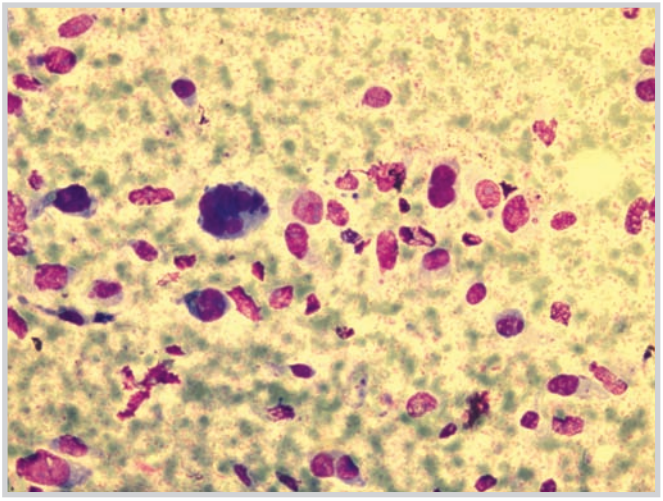

Fig. 36.5: Osteosarcoma: mononuclear, binuclear and multinuclear malignant cells showing moderate pleomorphism [MGG × medium power (MP)]

Compared to chondroid material, osteoid material is dense small aggregates. The presence of osteoid is essential for the diagnosis of osteosarcoma. In addition, the smear also shows discrete pleomorphic oval to spindle cells.

Conventional osteosarcomas are also divided into three types depending on the predominant matrix material, such as

1. Osteoblastic: In this type, osteoid matrix material is predominant component of the tumor stroma.
2. Chrondroblastic: In chondroblastic osteosarcoma, there is predominant chondroid material in the FNAC smear. Hyaline type of cartilage is intimately admixed with the tumor cells.
3. Fibroblastic osteosarcoma: In fibroblastic variant of osteosarcoma, the osteoid material is minimal in amount and the spindle cells are the predominant population in the smear. This tumor may simulate like fibrosarcoma or malignant fibrous histiocytoma (MFH).

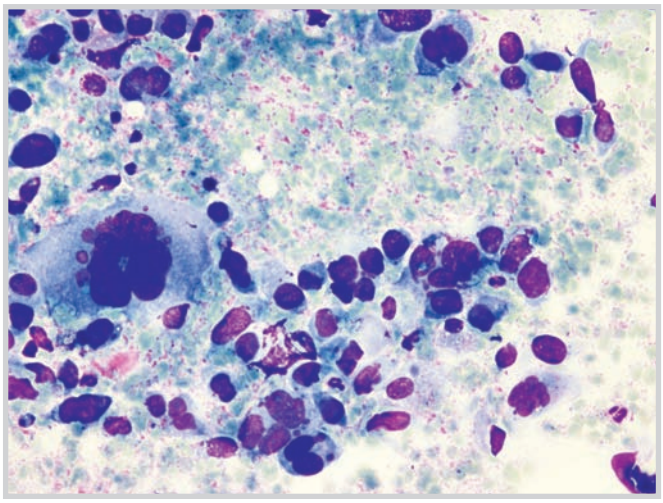

Fig. 36.6: Osteosarcoma: markedly pleomorphic bizarre cells and round pleomorphic cells in a reddish granular background (MGG × HP)

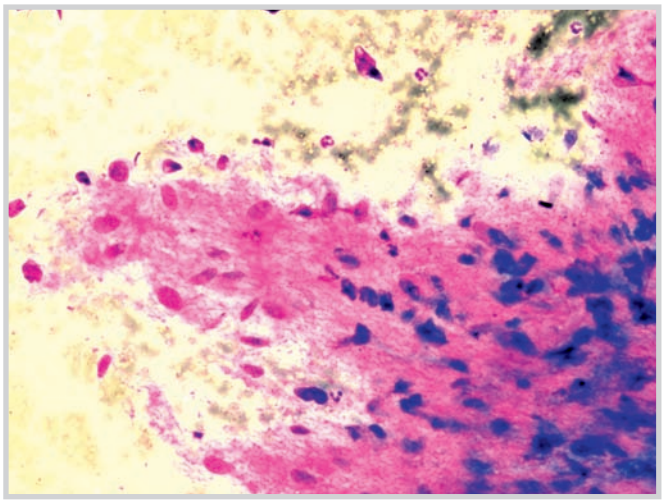

Fig. 36.7: Osteosarcoma: oval to elongated cells embedded in osteoid material (MGG × HP)

Small cell osteosarcoma: Small cell osteosarcoma is a special type of osteosarcoma that is composed of small cells with osteoid formation. It comprises 1.5% of osteosarcomas. The smear shows small round cells with scanty cytoplasm. These cells are four times larger than lymphocytes. The nuclei are round with fine granular chromatin. It resembles Ewing's sarcoma (EWS) of the bone.[17]

Telangiectatic osteosarcoma: Telangiectatic osteosarcoma is a rare subtype of osteosarcoma and it comprises less than 4% of all types of osteosarcoma. This tumor commonly arises in the distal femur and proximal tibia. The tumors are usually cystic filled with blood. FNAC yields hypocellular material. Smears show many multinucleated giant cells, atypical spindle cells and oval mononuclear malignant cells. The nuclei show marked pleomorphism. In addition, the smear may also show small epithelioid like cells.[18]

Central low-grade osteosarcoma: This tumor represents less than 1% of all primary bone tumors. They are difficult to diagnose on FNAC smear because of relatively low yield of material and low nuclear grade. Majority of the tumor is located in the long bone.

Radiographic picture of low-grade central osteosarcomas shows heavy mineralization and cortical bone destruction. This may simulate a fracture callus. FNAC smear shows oval to spindle-shaped nuclei with moderate pleomorphism along with osteoid material.

Differential Diagnosis

- *Malignant fibrous histiocytoma*: Fibroblastic type of osteosarcoma may pose diagnostic difficulty with MFH. However, the classical radiologic findings and the presence of osteoid favor osteosarcoma.
- *Ewing's sarcoma*: Small cell osteosarcoma may simulate EWS. The cells are more cohesive and nuclei show much more pleomorphism in osteosarcoma.

CARTILAGE FORMING TUMOR

Chondroma

This is a benign cartilaginous neoplasm and also known as enchondroma. This is a relative common benign tumor of bone and represents about one-fourth of all benign bone neoplasm. Enchondroma may occur in any age group; however, this is commonly seen in 20–30 years age. The common sites of enchondroma are short tubular bones of hand and foot. Long tubular bones like humerus and femur are also involved. The patients of enchondroma of long tubular bone are usually asymptomatic. Enchondroma of short tubular bone presents as swelling and pain. X-ray of enchondroma shows well-circumscribed radiolucent or heavily mineralized bony swelling.

Cytology

Fine needle aspiration cytology smears of enchondroma show predominantly very thick hyaline cartilage with background metachromatic material (**Box 36.5**). The individual cells are embedded in the cartilaginous material (**Fig. 36.8**). The nuclei are round with inconspicuous nucleoli. The cells do not show any binucleation. Mitotic activities are absent. Cytology smear of enchondroma and low-grade chondrosarcoma (CHS) is difficult to differentiate.

Chondroblastoma

This is a benign cartilaginous neoplasm of bone and comprises less than 1% of all bone tumors. The age of the patient varies from 10 years to 25 years. The lesion typically occurs in the epiphyseal and epimetaphyseal regions of long bones particularly femur, tibia and humerus. The patient complains of localized pain, swelling and restricted joint movement (in case the lesion occurs near the joint). X-ray of the chondroblastoma shows a sharply demarcated lytic lesion with a sclerotic outer rim.

BOX 36.5 Enchondroma

- Hyaline cartilage
- Metachromatic material
- Round cells
- Single round bland nuclei

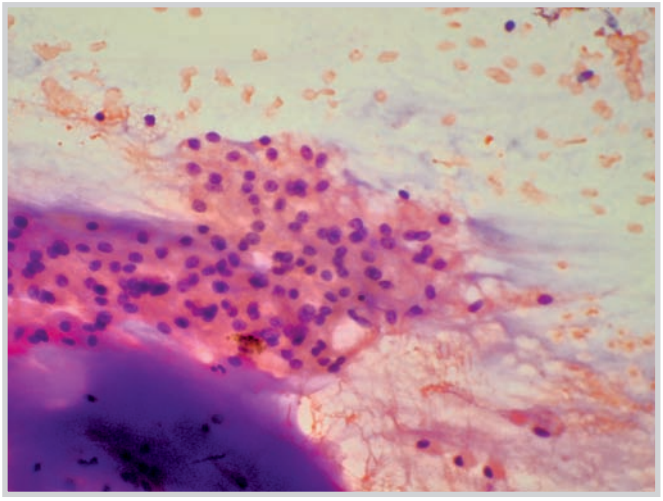

Fig. 36.8: Enchondroma: benign chondrocytes in the background of thick cartilaginous material (H & E × HP)

BOX 36.6 Chondroblastoma

- Chondroblasts: polygonal to spindle cells
- Oval nuclei
- Fine chromatin
- Longitudinal nuclear groove
- Many osteoclastic giant cells
- Chondroid material

Cytology

Fine needle aspiration cytology of chondroblastoma shows many chrondroblasts, giant cells and cartilaginous material (**Box 36.6**). The primitive chondroblasts are present as poorly cohesive or discrete round shaped cells. The margin of the cell is well-defined. The cytoplasm of the cell is dense with monomorphic round nuclei. The nuclear chromatin is fine. Nuclei of the cells show deep longitudinal groove. Occasional cells may show intranuclear inclusion. The smear shows abundant multinucleated osteoclastic giant cells.

These osteoclastic giant cells usually contain 5–20 nuclei. Occasionally, large number of nuclei as many as 60 nuclei can be seen. Unlike round nuclei of the mononuclear cells, these nuclei are ovoid in appearance. The smears also show amorphous or fibrillar matrix material. This appears as purplish color in MGG smear.[19,20]

Chondrosarcoma[21-25]

This is a malignant tumor with cartilaginous differentiation. CHS represents 20% of all malignant bone tumors. The primary CHS commonly occurs in adults above 50 years of age. The most common sites of primary CHS are flat bones of the pelvis, proximal femur, proximal humerus, distal femur and ribs. The patient usually presents with longstanding pain and swelling. Radiological examination of CHS shows expansile radiolucent lesion with cortical thickening. The lesion often shows cortical erosion or destruction.

Cytology (Figs 36.9 to 36.11)

Chondrosarcoma are graded as grade I, grade II and grade III. The grading of CHS is based on nuclear size, nuclear hyperchromasia and cellularity. Cytological grade is well-correlated with histological grade. FNAC of CHS yields gelatinous or myxoid material (**Box 36.7**). The smears show reddish purple cartilaginous material in MGG stain. Depending on grade of CHS, the amount of the cartilaginous material varies. Low-grade CHS shows abundant cartilaginous material compared to high-grade CHS. Similarly, the cellularity also increases from low-grade CHS to high-grade CHS. Tumor cells lie predominantly singly in chondroid or myxoid material. The tumor cells are large with well-defined cytoplasmic border. Cytoplasm is pale and vacuolated. Nuclei of the cells are central in position and round in shape. Nuclear enlargement, pleomorphism and hyperchromasia increase with increasing grade. The tumor cells frequently show binucleation. Mitosis is usually absent. However, necrosis is frequently present in high-grade CHS. Abundant myxoid change is more frequently seen in high-grade CHS. However, fragments of thicker more hyaline cartilage are seen in grade II CHS.

There are various other types of CHS such as mesenchymal CHS, clear cell variety, and dedifferentiated CHS.

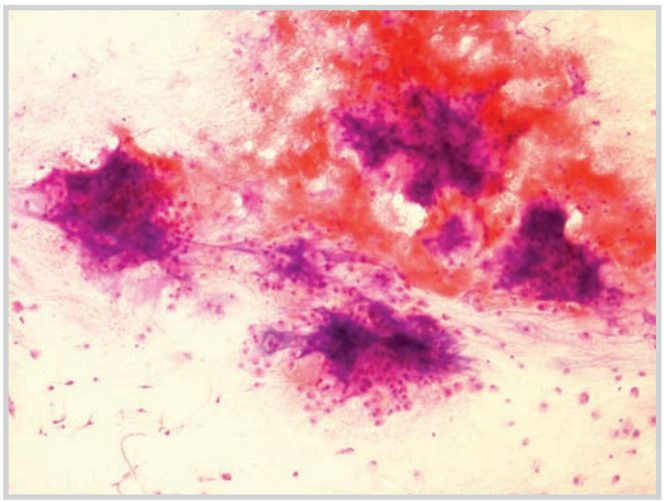

Fig. 36.9: Chondrosarcoma: abundant chondrocytes embedded in cartilaginous material [H & E × low pressure (LP)]

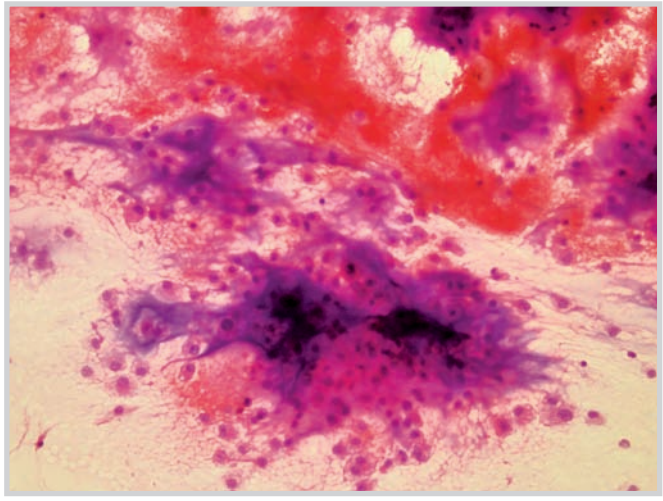

Fig. 36.10: Chondrosarcoma: chodrocytes with moderate cytoplasm and monomorphic looking nuclei. However, many binucleated chondrocytes are seen (H & E × MP)

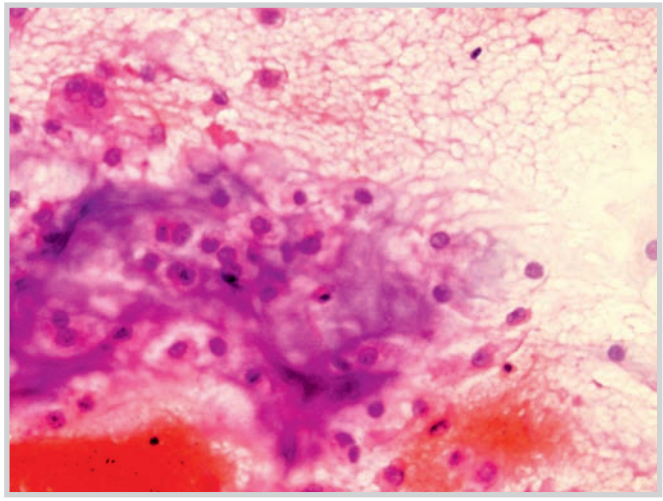

Fig. 36.11: Chondrosarcoma: higher magnification showing binucleated chondrocytes (H & E × HP)

BOX 36.7 Chondrosarcoma

- Chondroid or myxoid material
- Isolated cells
- Large cell with well-defined border
- Pale foamy cytoplasm
- Enlarged pleomorphic and hyperchromatic nuclei
- Binucleated cells

Mesenchymal Chondrosarcoma

It is a rare malignant cartilaginous tumor and represents less than 3% of all primary CHS. The tumor affects all age groups; however, this is more frequent in second and third decades of life. The common sites of involvement are jaw bones, ribs, pelvic bones and vertebra. The patient usually complains of pain and swelling. Radiological examination of the tumor usually shows an osteolytic lesion with destruction of cortical bone and extension of the tumor in the adjacent soft tissue.

BOX 36.8 Mesenchymal chondrosarcoma

- Cartilaginous material
- Osteoclast-like giant cells
- Small round blue cells
- High many osteoclasts nucleus-to-cytoplasm (N/C) ratio

BOX 36.9 Clear cell chondrosarcoma

- Large cells
- Clear cytoplasm
- Central nuclei
- Multinucleated giant cells

Cytology

The tumor is biphasic in pattern on histology with admixture of small round undifferentiated cells and hyaline cartilage. The FNAC smears are usually rich in cells. The cells are small round with scanty cytoplasm and round nuclei with coarse chromatin. Nucleus-to-cytoplasm (N/C) ratio of the cell is high. In addition, the smear also shows many osteoclasts-like giant cells and cartilaginous material (**Box 36.8**). Background of the smear may show necrosis and hemorrhage.

Clear Cell Chondrosarcoma

It is a rare variety of CHS that represents 2% of all CHS. The tumor may occur in any age group. Clear cell CHS commonly affects skull, spine, hands and feet. FNAC of this tumor shows discrete large cells with clear cytoplasm and centrally placed nuclei (**Box 36.9**). Many multinucleated giant cells are seen. In addition, cartilaginous material is also seen.

Dedifferentiated Chondrosarcoma

This is a type of CHS that contains two types of component: (1) well-differentiated CHS and (2) high-grade noncartilaginous sarcoma. The tumor represents about 10% of all CHS. The tumor commonly affects in fifth and sixth decade of life. The common sites of dedifferentiated CHS are pelvis, femur and humerus. Cytology smear shows chondrocytes admixed with cartilaginous material along with the cells of poorly-differentiated sarcoma.

Diagnostic Difficulties

Enchondroma: It is very difficult to differentiate enchondroma from a low-grade CHS. Radiographic picture of cortical

disruption along with high cellularity cellularity and binucleated cells indicate the possibility of CHS.

Giant Cell Tumor of Bone[26-28]

Giant cell tumor (GCT) of bone is a benign but locally aggressive tumor that represents 5% of all bone tumors. This is usually seen over the age of 18 years with the maximum incidence between 25 years and 45 years. GCT predominantly involves the distal femur, proximal tibia, distal radius and proximal humerus. Radiologically, the tumor represents the expanding epiphyseal osteolytic lesion. Occasionally, the tumor produces a trabeculated "soap-bubble" appearance.

Cytology (Figs 36.12 to 36.14)

Cytology smear shows cluster and discrete abundant multinucleated osteoclastic type of giant cells and mononuclear oval to spindle cells (**Box 36.10**). The overall arrangement of cluster and sheets of cells gives a checkerboard like appearance. The osteoclastic giant cells are relatively large with abundant cytoplasm containing 50–100 nuclei. These cells are intimately mixed with the stromal cells. The stromal cells have moderate cytoplasm and oval to spindle-shaped nuclei with fine granular chromatin and distinct nucleoli. The nuclear margin is smooth. The mononuclear cells often show syncytial appearance when they are present in clusters. The nuclear character of the giant cells and the stromal cells are similar. It is now proved beyond doubt that the stromal cells are the neoplastic component of GCT. These cells often show increased mitotic activity; however, atypical tripolar mitosis is rarely seen and indicates the presence of sarcoma with giant cell reaction.

Differential Diagnosis

- *Other bone lesions rich in osteoclastic giant cells*: GCT of bone should be differentiated from the other bony lesions rich in osteoclastic giant cells that include osteosarcoma, chondroblastoma, ABC and brown tumor of hyperparathyroidism. Pleomorphic and hyperchromatic nuclei of the osteosarcoma help in differentiating it from GCT. The stromal cells of chondroblastoma are round to oval with longitudinal deep nuclear groove. ABC and brown cyst show many hemosiderin laden macrophages.

Chordoma[29-31]

Chordoma is a low to intermediate grade malignant tumor that develops from the remnants of notochordal rest and occurs in both ends of the neural axis. It represents 2–4% of all malignant bone tumors. Chordoma is commonly seen in middle aged to elderly patients and rarely occurs before 30 years of age. The commonest location of the tumor is sacral and spheno-occipital region.

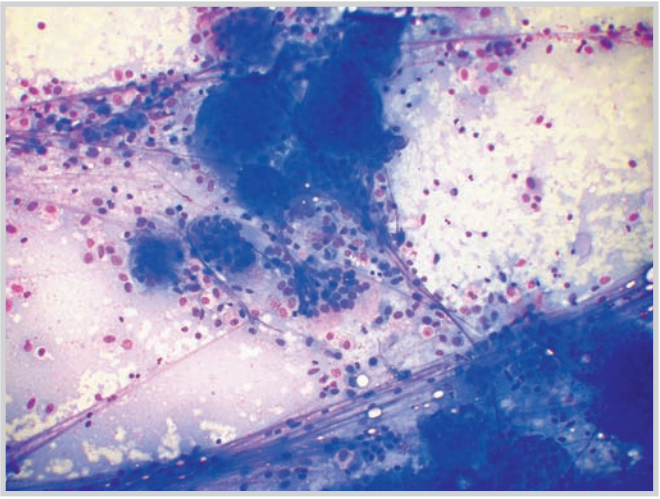

Fig. 36.13: Giant cell tumor of bone: clusters of osteoclastic giant cells along with stromal cells (MGG × MP)

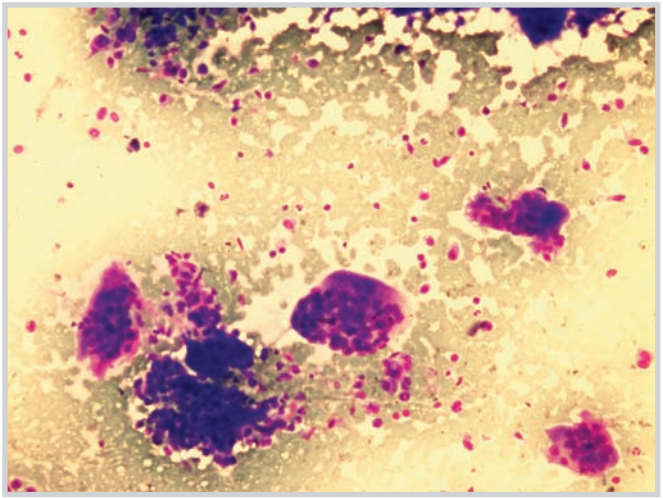

Fig. 36.12: Giant cell tumor of bone: multiple osteoclast-like giant cells along with oval to spindle-shaped stroma (MGG × MP)

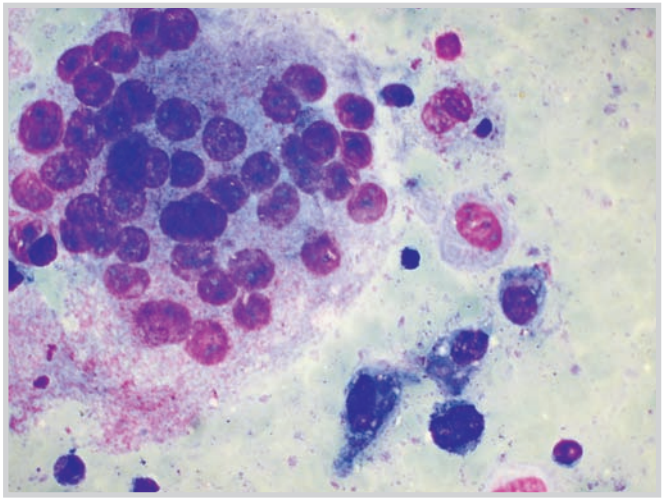

Fig. 36.14: Giant cell tumor of bone: higher magnification showing better morphology of the giant cells and stromal cells (MGG × HP)

BOX 36.10 **Giant cell tumor of bone**

- Abundant multinucleated osteoclastic giant cells
- Mononuclear ovoid to spindle cells
- Giant cells are intimately attached with spindle cell clusters

Cytology (Figs 36.15 to 36.18)

Fine needle aspiration cytology of chordoma yields myxoid or mucoid material. The smear gives a reddish purple color appearance on MGG stain (**Box 36.11**). The bimorphic population of cell is embedded in the matrix material. There are two types of cells: (1) large cells and (2) relatively small mononuclear granular cells. The larger cells have abundant vacuolated bubbly cytoplasm which gives a basket-like appearance. These cells are also known as *physaliphorous* cells and are characteristic cells of chordoma. The nuclei of the cells are small round monomorphic and central in position. N/C ratio of the cell is low. Nuclear chromatin is granular with prominent nucleoli. The most of the cells are mononuclear; however, binucleated and multinucleated cells are also seen. Mitotic activities are absent.

The other cells are relatively small with modest amount of granular cytoplasm. The nuclei are round and monomorphic with prominent nucleoli. Intranuclear cytoplasmic inclusions may also be noted. Characteristically, chordomas are positive for S-100 protein, cytokeratin (CK) and epithelial membrane antigen (EMA) whereas, CHS cells are S-100 positive and negative for CK.

Differential Diagnosis

- *Chondrosarcoma*: The classical physaliphorous cells are never present in CHS. The background myxoid material encircling the individual cells are also characteristic of chordoma.
- *Metastatic adenocarcinoma*: Renal cell carcinoma may show clear vacuolated cell and simulate chordoma. However, the nuclei of these cells are usually enlarged and pleomorphic and the smears show lack of the typical myxoid background. Adenocarcinoma cells are negative for S-100 and positive for CK.

Ewing's Sarcoma[32-34]

Ewing's sarcoma is a malignant round cell tumor with neuroectodermal differentiation and commonly occurs in the age group of 5–25 years. EWS typically shows specific t(11;22)(q24;q12) chromosomal translocation. EWS represents about 5% of all primary malignant bone tumors. It commonly arises from the diaphysis or metaphyseal-diaphyseal part of the long bone particularly from femur and tibia. Radiographically, the tumor shows an osteolytic lesion and long bone shows onion skin like periosteal reaction. The tumor often involves the soft tissue and presents as a soft tissue mass.

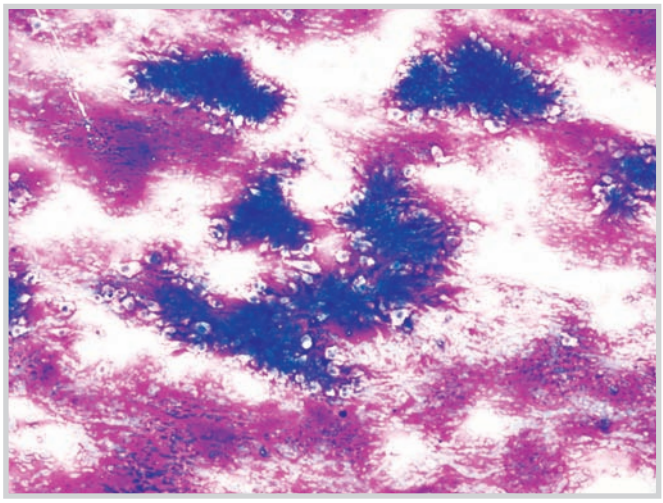

Fig. 36.15: Chordoma: multiple clusters of vacuolated cells in abundant pinkish material (MGG × MP)

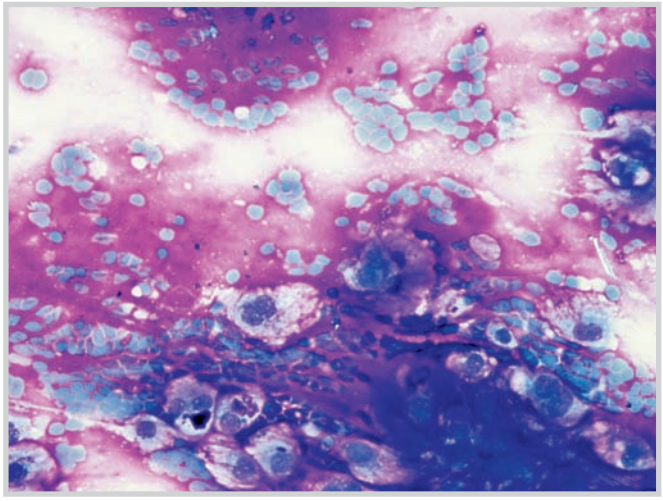

Fig. 36.16: Chordoma: physaliphorous cells showing clear multivacuolated cytoplasm and centrally placed nuclei (MGG × HP)

Cytology (Figs 36.19 and 36.20)

Fine needle aspiration cytology smear of EWS shows bimodal population of light colored and dark colored small round cells (**Box 36.12**). The tumor cells are discrete round cells with scanty deep blue cytoplasm. Cytoplasmic vacuolations are often seen due to the presence of glycogen. Nuclei are round with high N/C ratio and inconspicuous nucleoli. Nuclear molding may also be seen. However, typical crushing artifact is absent. Occasional pseudorosette like structures may be seen. The smears may also show necrosis and perivascular arrangement of cells.

The cells of EWS are positive for CD99 (Mic-2). The tumor cells are also positive for vimentin and neuron specific enolase (NSE).

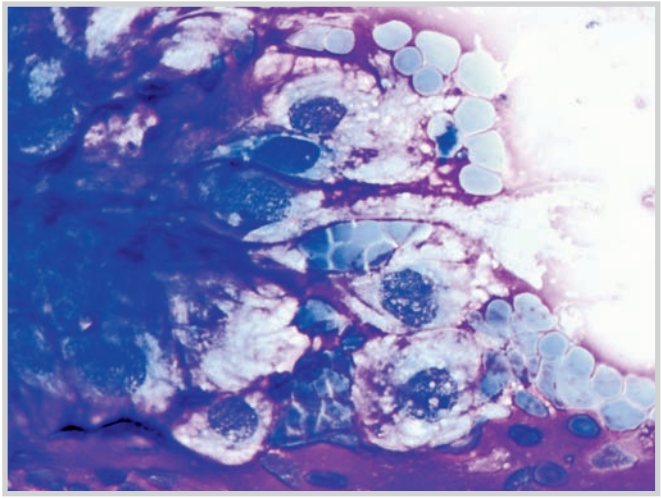

Fig. 36.17: Chordoma: higher magnification showing better morphology of the physaliphorous cells. Note the typical bubbly vacuolated cytoplasm [MGG × oil immersion (OI)]

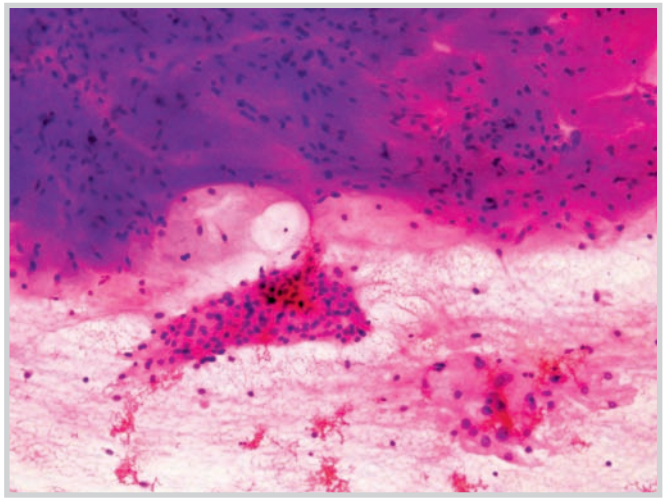

Fig. 36.18: Chordoma: smaller round and monomorphic cells (H & E × MP)

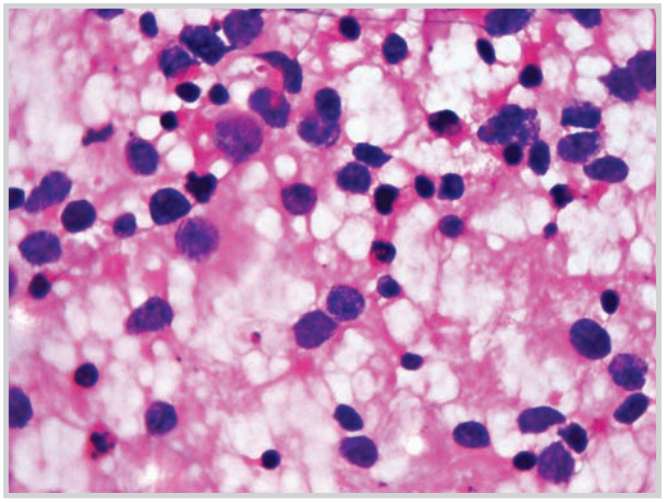

Fig. 36.19: Ewing's sarcoma: discrete small round cells with scanty cytoplasm. Nuclei are monomorphic with granular chromatin having inconspicuous nucleoli (H & E × HP)

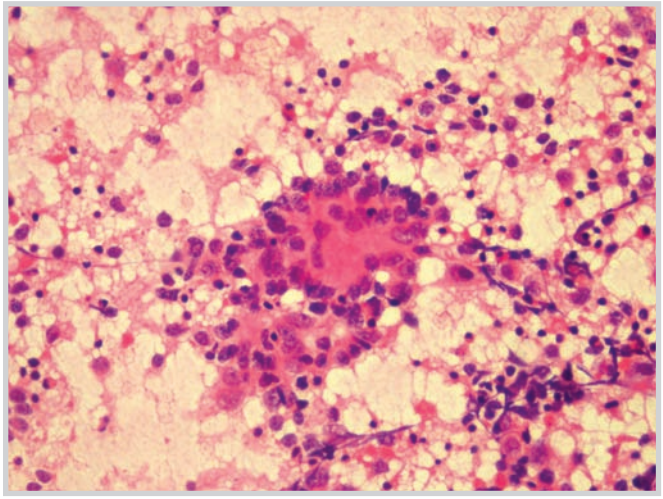

Fig. 36.20: Ewing's sarcoma: rosette-like structure (H & E × HP)

BOX 36.11 Chordoma

- Abundant myxoid material
- Large cells (physaliphorous cells) with abundant bubbly vacuolated cytoplasm and central round nuclei
- Relatively smaller mononuclear cells with moderate granular cytoplasm and round nuclei
- S-100 positive and negative for cytokeratin (CK)

Diagnostic Difficulties

Other small round cell tumor: Osteosarcoma of small cell type and non-Hodgkin lymphoma (NHL) is the differential diagnoses of EWS of the bone.

BOX 36.12 Ewing's sarcoma

- Discrete small round cells
- Bimodal cells: Light and dark cells
- Dark cells show scanty deep blue cytoplasm
- Light cells show moderate vacuolated cytoplasm
- Round monomorphic nuclei
- Fine granular chromatin
- Inconspicuous to absent nucleoli
- Rosette-like structures may be seen
- CD99 (Mic2) positive. Variable positivity of vimentin, NSE and CK
- t(11;22) (q24;q12) chromosomal translocation

Abbreviations: NSE, neuron specific enolase; CK, cytokeratin

BOX 36.13 Non-Hodgkin's lymphoma of bone

- Predominantly diffuse large B-cell (DLBC)
- Occasionally, follicular center cell lymphoma and anaplastic large cell lymphoma
- Seven percent of all bone malignancies
- Flow cytometric immunophenotyping is needed

Non-Hodgkin Lymphoma

This may involve the bone secondarily. NHL of bone represents 7% of all bone malignancies. The primary NHL of bone is rare. Lymphoma commonly affects femur followed by spine and pelvic bones. The patient commonly complains of pain or swelling of the bone. More than 90% of primary lymphoma of bone in adult is diffuse large B-cell lymphoma (DLBCL) (**Box 36.13**). Rarely follicular center cell lymphoma and anaplastic large cell lymphoma may occur. The patients of primary bone lymphoma present with bone pain. The patients may have (1) single site of involvement of lymphoma with or without lymph node involvement, (2) multiple skeletal sites of involvement with or without any visceral involvement, and (3) prior history of lymphoma with bone involvement in biopsy sample. The cytomorphology of NHL of bone is same as that of other body sites. Flow cytometric (FCM) may be needed for exact diagnosis and classification of NHL of bone.

Plasma Cell Neoplasm[35-37]

Plasma cell myeloma (PCM) is the neoplasm of plasma cells. It is also known as multiple myeloma. This is the most common bone tumors. The tumor usually occurs in the 6th decade. PCM commonly affects vertebrae, ribs, skull, pelvis and femur. The patient usually complains of pain and pathological fracture of bone along with symptoms of hypercalcemia and anemia. Radiographic examination of the bony lesion shows a sharply demarcated lytic area surrounded by sclerosis.

Plasma cell neoplasm may be solitary presenting as plasmacytoma or disseminated plasma cell proliferation such as multiple myeloma.

The major and minor criteria of multiple myeloma are described in **Box 36.14**.[38]

Cytology (Figs 36. 21 and 36.22)

Fine needle aspiration cytology smear of PCM shows abundant discrete round to oval cells of plasma cell lineage with variable cellular maturity (**Box 36.15**). The well-differentiated plasma cell tumors show normal looking plasma cells. The individual cells are round with moderate to abundant amount of dense eosinophilic cytoplasm in hematoxylin and eosin (H & E) staining. The nuclei are eccentric and show peripheral clumped chromatin giving rise to cart wheel appearance. In MGG stained smears, the cytoplasm is basophilic with a perinuclear clear halo. This is due to rough endoplasmic reticulum and prominent Golgi center. Binucleated and occasionally multinucleated plasma cells are also seen.

BOX 36.14 Criteria of multiple myeloma

Major criteria:
- Plasmacytoma on biopsy
- Marrow plasmacytosis: more than 30% plasma cells
- Presence of myeloma component:
  - Serum IgG > 3.5 g/dL, IgA > 2 g/dL
  - Urine > Bence Jones protein without amyloidosis.

Minor criteria:
- Marrow plasmacytosis (10–30% plasma cells)
- Myeloma component presents but less than listed above
- Lytic bone lesions
- Reduced normal levels of immunoglobulins

(<50% normal: IgG <600 mg/dL, IgA 100 mg/dL, IgM <50 mg/dL)
Multiple myeloma: (1) One major and one minor criterion or (2) At least three minor criteria

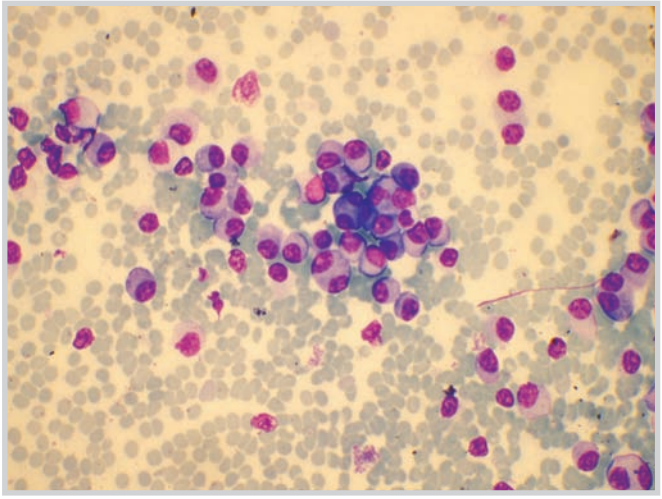

Fig. 36.21: Plasmacytoma in bone: clusters of plasma cells. Many cells are binucleated (MGG × MP)

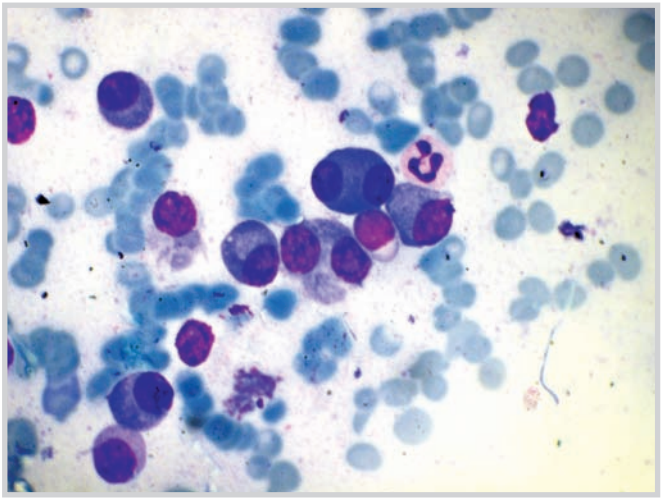

Fig. 36.22: Plasmacytoma in bone: higher magnification showing better morphology of the plasma cells (MGG × OI)

BOX 36.15 Plasma cell myeloma

Well-differentiated
- Round cells
- Abundant cytoplasm
- Eccentric nucleus
- Cart wheel chromatin (peripheral clumped chromatin)
- Perinuclear halo

Poorly differentiated
- Plasmablasts
- Round cells
- Scanty cytoplasm
- Fine chromatin
- Prominent nucleoli

Poorly-differentiated plasma cell tumor has poor prognosis. FNAC smear shows many plasmablasts. These cells have scanty amount of cytoplasm, high N/C ratio, opened up chromatin and prominent nucleoli. Binucleated and multinucleated cells are more frequent. In addition, many atypical mitotic figures are also noted. A careful search always reveals typical plasma cells.

Diagnostic Difficulties

Reactive plasma cells: It is always necessary to differentiate reactive plasmacytosis from PCM. Reactive plasmacytosis may occur secondary to chronic infection, metastatic tumor in bone or due to reaction to primary bone tumor. Strict major and minor criteria of diagnosis are helpful in this occasion.

Langerhans Cell Histiocytosis[39,40]

Langerhans cell histiocytosis (LCH) is the neoplasm of Langerhans cells and represents 1% of all bone tumors. LCH commonly occurs in young patients under 10 years. However, it may occur in patients of any age groups. LCH commonly involves the skull, pelvis and femur. The patient usually complains of pain and swelling; however, the symptoms largely depend on the sites of involvement of bone.

Cytology (Fig. 36.23)

Aspirates show discrete population of Langerhans cells. The cells are large with abundant cytoplasm and well-defined cell border (**Box 36.16**). The nuclei are large, round-shaped with indented margin and characteristic longitudinal nuclear groove. Nuclear chromatin is dispersed or condensed with occasional prominent nucleoli. Background of the smear shows eosinophils, lymphocytes, polymorphs and macrophages. In addition, large osteoclastic giant cells and histiocytes with phagocytosed materials are also noted.

Immunocytochemistry

The cells of LCH are negative for CD45. They characteristically express CD1a and S-100 protein.

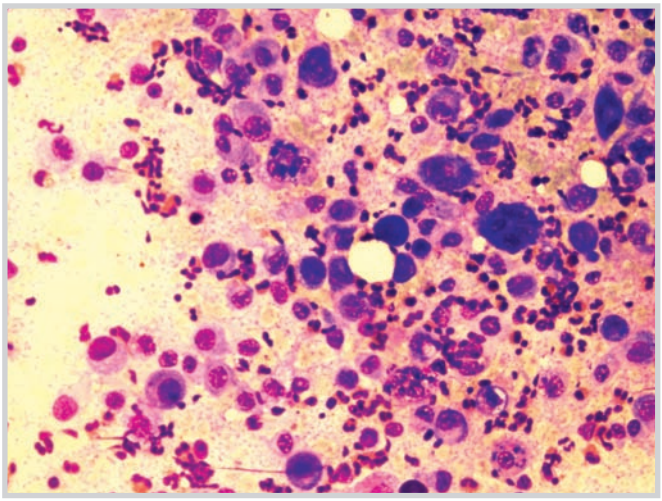

Fig. 36.23: Langerhans cell histiocytosis: many mononuclear and multinuclear histiocytes with deep nuclear groove along with abundant eosinophils (MGG × MP)

BOX 36.16 Langerhans cell histiocytosis

- Large cells with abundant cytoplasm
- Round nuclei with longitudinal grooves
- Many bi and multinucleated cells
- Eosinophils
- Lymphocytes, polymorphs and macrophages

Immunocytochemistry: CD1a positive
Electron microscopy: Birbecks granules

Differential Diagnosis

Fungal infections: Large number of eosinophils and histiocytes may mislead the diagnosis of fungal infection. However, the characteristics Langerhans cell with deep longitudinal grooves, absence of fungal structure and clinical features may help in the diagnosis.

Metastatic Carcinoma

Metastatic carcinoma is the most frequent malignant tumor of the bone. Majority of the patients are between 40 years and 60 years of age. The common sites of origin of malignancies are breast, lung, prostate, kidney and thyroid. The metastasis commonly affects axial skeleton such as vertebra, ribs, sternum, pelvis and skull bones. In most of the cases, the clinical history or thorough radiographic examination helps to identify the primary site of origin of carcinoma. Occasionally, solitary bone lesion from an unknown primary site may mimic as primary bone tumor.

Cytology (Figs 36.24 and 36.25)

Cytomorphology of the individual cells depend on the type of primary malignancy. The metastatic tumor often simulates the morphology of the primary malignancies and these foreign cells are easily recognizable. Gland-like arrangement is seen

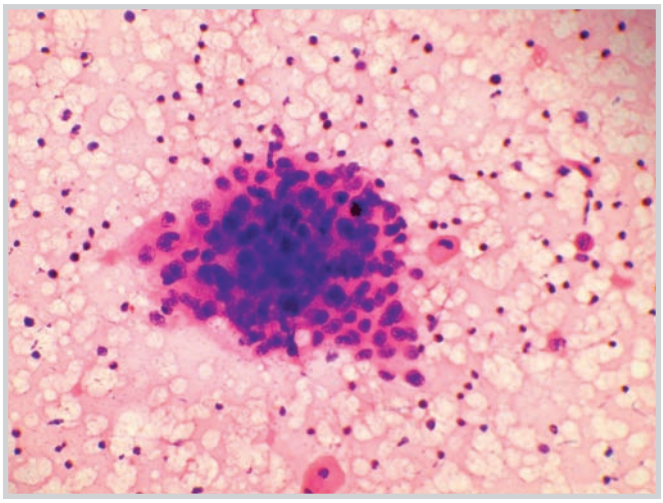

Fig. 36.24: Metastatic carcinoma: tight cluster of malignant cells in a metastatic prostatic carcinoma in bone (H & E × MP)

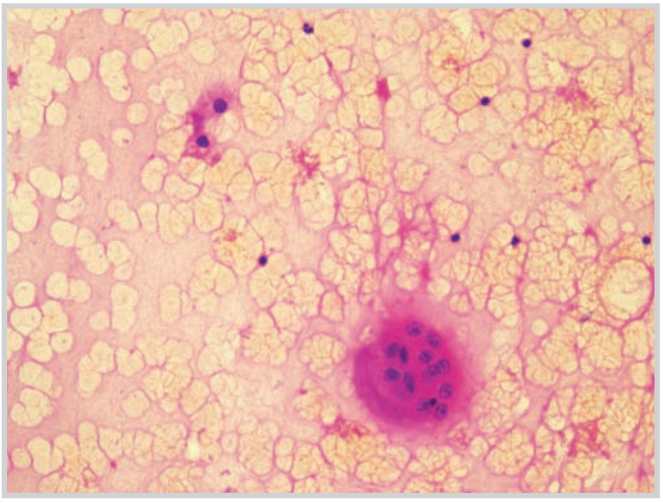

Fig. 36.26: Aneurysmal bone cyst: multinucleated giant cells and foamy histiocytes (MGG × MP)

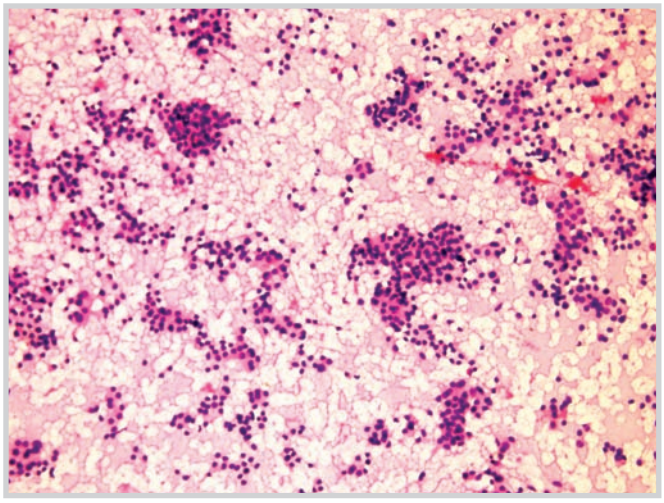

Fig. 36.25: Metastatic follicular cell carcinoma: discrete and follicular arrangement of cells in metastatic follicular carcinoma of thyroid (H & E × LP)

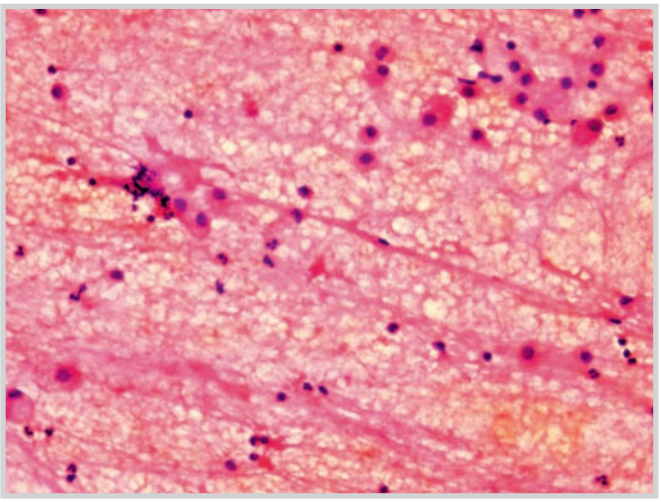

Fig. 36.27: Aneurysmal bone cyst: many foamy histiocytes are seen in the background (MGG × MP)

in case of adenocarcinoma. Multiple follicular arrangements of cells are noted in metastatic follicular carcinoma of thyroid. Squamous cell carcinoma shows polyhedral squamoid cells with orangeophilia. Fiber and tadpole cells are also noted. Renal cell carcinoma shows large cells with clear cytoplasm and centrally placed enlarged nuclei. Metastatic spindle cell type of renal cell carcinoma may mimic sarcoma of the bone. Panel of immunocytochemistry may be helpful in difficult situation.

Aneurysmal Bone Cyst

Aneurysmal bone cyst is a benign cystic lesion of bone. It commonly occurs in the second decade of life. ABC may occur as a primary bone lesion or secondary to other benign or malignant tumors such as GCT of bone, chondroblastoma, fibrous dysplasia, osteoblastoma, eosinophilic granulomas and osteosarcomas.[41,42]

ABC may occur in any bone; however, it commonly affects the metaphysis of long bones such as femur, tibia and humerus, and the posterior part of vertebral bodies.

Cytology (Figs 26 and 36.27)

Fine needle aspiration cytology of ABC yields predominantly blood. The smears show many hemosiderin laden histiocytes, fragments of fibroconnective tissue and multiple osteoclastic giant cells (**Box 36.17**). Frequent mitosis may be present. Necrosis is usually absent; however, it may be seen in case of pathological fracture of bone.

Adamantinoma

Adamantinoma is a rare low-grade biphasic malignant tumor of bone that represents less than 0.5 % of primary bone tumors. It usually occurs in young patient of 25–30 years of age. More than 90% adamantinoma occurs in the shaft of tibia. Radiograph shows a well-circumscribed, cortical, osteolytic lesion with sclerosis in the circumference.

Cytology

Fine needle aspiration cytology of adamantinoma shows multiple clusters of malignant epithelial cells, fibroblastic stromal cells and squamous-like cells (**Box 36.18**). The tumor cells are arranged in clusters with peripheral palisading arrangement. The individual cells are round to oval with monomorphic nuclei and bland chromatin.

BOX 36.17 Aneurysmal bone cyst

- Bloody smear
- Pigment laden histiocytes
- Osteoclastic giant cells

BOX 36.18 Adamantinoma

- Malignant epithelial cells
- Palisading arrangement of cells
- Cells with high N/C ratio
- Monomorphic nuclei
- Squamoid cells in nest
- Scattered fibroblasts

Differential Diagnosis

- *Metastatic carcinoma*: Malignant epithelial cells may be mistaken as metastatic carcinoma. However, the young age of the patient, typical location of the tumor in tibial shaft and relatively monomorphic nuclei help in the diagnosis of adamantinoma.

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